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LIFE CYCLES IN BACTERIA[1]

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Life cycles are a law of nature. In the highest forms of life the changes are obvious because they are gradual, and the individuals are so large that transformations may be readily followed. On the other hand, in lower forms of life the transformations may be abrupt and therefore difficult to follow. In microscopic organisms the changes are particularly difficult to follow because continuous observation of an individual organism and of its progeny requires great patience and skill. In single-celled organisms the parent cell may be of one form, and all its descendants may be of another form.

Presumably it was on account of the difficulties in following bacterial changes that the monomorphic theory was adopted. No doubt the investigations of the early bacteriologists were more productive of practical knowledge under the monomorphic theory than would have been possible under a pleomorphic theory, for the monomorphic theory held in check the confusion which would have arisen in the new science if attention had been diverted to the intricate problems of life cycles. No science has a more brilliant decade's record than was made by bacteriology, particularly by medical bacteriology, in the 80's under the monomorphic theory.

Bacteriology cannot progress, however, if original theories are held tenaciously when evidence accumulates against them. Chemists, physicists, geologists and astronomers are continually altering theories to fit newly discovered facts. Bacteriologists must do likewise or their science will lag. A constantly increas-

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ing amount of evidence is accumulating against the theory of monomorphism in bacterial organisms. It is the purpose of this paper to examine this evidence in the light of the general law of life cycles as illustrated by familiar organisms standing higher than bacteria in the scale of life.

A brief survey of the general law of life cycles may be obtained by considering their occurrence in three widely separated groups—(1) vertebrates, (2) insects, (3) fungi and algae. These groups include representatives of the highest, an intermediary and the lowest forms of life, and they include groups from both the animal and the plant kingdoms.

Some bacteriologists have expressed their inability to accept the theory of bacterial pleomorphism because they say that if they accepted pleomorphism, they would also have to accept complete mutation in biologic behavior. When changes of form have been reported, they involved one or more other changes as, for example, changes in habitat or in physiologic behavior, or, in the case of pathogenic bacteria, changes in pathogenicity or in immunologic relationships. The fact that bacteria of a given form may be maintained indefinitely in culture media presents another difficulty. If bacteria undergo life cycles, why is it that certain cultures have been maintained for many years, even for many decades, without a change of form?

It is in order to consider some of these perplexities from the point of view of general biology that the examples of life cycles to be cited are chosen. From them several deductions may be made: (1) Life cycles are a law of nature. (2) Changes in morphology may be abrupt. (3) Changes in morphology are accompanied by changes in biologic behavior. (4) Environment conditions the development of inherited characters. (5) There may be irregularities in the life cycle. (6) The reproduction of a single phase of the cycle may continue relatively indefinitely. (7) Irregularities in life cycles depend on external influences.

The cycle in man, and in most other vertebrates, proceeds in regular order. The differences in the characteristics of the young and the adult are considerable, however, even in man. Suppose that our planet were to be visited by an intelligent being

from Mars, and that the Martian, although of a form entirely different from man, possessed the same kind of mental faculties, and that he had developed his faculties along the same lines as a bacteriologist. If his first view of human beings were limited to two individuals, a mother and her young infant, he would probably think he had found two species, one parasitic upon the other, for the two would not look alike, they would not act alike, one would be twenty times the size of the other, and most important to his bacteriological mind, the two individuals would not eat the same kinds of food. If the Martian would prolong his visit, however, so that he could observe a large number of human beings, he would find every gradation in size and behavior between the extremes of mother and child. He would have to modify his preliminary hypothesis and conclude that the two individuals represent different stages in the life cycle of one species.

Although gradual changes are the rule in vertebrates, sudden changes, called metamorphoses, occur in certain species of that group, as for example, in amphibians and the lamprey. In the frog, metamorphosis occurs at the end of the aquatic or tadpole stage. At this time there is a sudden bursting into function of organs which developed gradually during the previous tadpole life. There are structural changes in the organs of the respiratory and vascular systems, and the paired limbs appear. These changes are required for the terrestrial life of the adult.

In the Hexapoda, or insects, metamorphoses, or sudden changes, are the general rule. Most insects pass through a life cycle of four distinct phases—egg, larva, pupa and adult. In certain species of insects, however, there are notable irregularities in the cycle. A brief review will be given of the life history of the rosy aphid (*Anuraphis roseus*), an insect whose life history exhibits a variety of forms and remarkable irregularities depending on environment. Eggs of the rosy aphid laid on the apple tree in the fall hatch at about the time the buds break in the spring. The aphids hatching from the winter eggs are called stem-mothers. The stem-mother matures in about fifteen days, whereupon she begins producing young at an average of six a day. She lives

from a month to six weeks. Her offspring are of a rosy color and may be either winged or wingless. The production of these forms on the apple continues for several generations. When mature, the winged forms fly to plantains where they produce young throughout the summer months. Most of the aphids produced upon the plantain are wingless, although a few winged ones occur throughout the summer. Generation after generation develops on the plantain. Their color is yellowish green. As autumn approaches winged forms, consisting of both males and gamogenetic females, appear on the plantain. They are able to live and reproduce only upon the apple or closely related species. These fall migrants mate and produce on the apple leaves wingless, orange-yellow females, which complete the cycle by laying the winter eggs.

There are other species of aphids, which have life histories even more strange than that of the apple aphid. In *Aphis rosae*, the plant-louse of the rose, the young may be born by the oviparous or by the viviparous method, and either gamogenetically or parthenogenetically, and they may develop into winged forms or remain wingless. If the food plant is grown in a conservatory with protection against cold, the rose aphid may go on reproducing parthenogenetically without cessation for many years.

Another familiar example from insect life illustrates strikingly how external influences determine the development of one or another form of an organism. The kind of food supplied to the larvae of bees determines whether the females shall be fertile queens or infertile workers. The marked differences in structure and instincts of the two classes of females are all conditioned by the food provided for the larvae. Each larva inherits the capacity to react in either way according to the stimulus received. Other examples from various animal and plant species might be cited to show how conditions in respect of light or moisture, as well as food or temperature, may determine the form which an organism will take.

In the algae and fungi, those closely related groups of organisms standing next above bacteria, life histories are extremely complex. Thousands of species of fungi are classed as *Fungi Im-*

perfecti, because the members are supposed to be stages in the life cycles of forms belonging to the Ascomycetes and Basidiomycetes. The final classification of the members of the *Fungi Imperfecti* must await a knowledge of their life histories.

Certain of the fungi having exceedingly complex life cycles have been carefully studied on account of their great economic importance. Among these are the Uredinae, or rust fungi, which attack grasses and other plants of economic importance. In certain of the rust fungi five different kinds of spores may develop in different stages of their life history. Over one hundred and fifty species of rust fungi are known to be heteroecious—that is, dependent on two distinct host plants for the completion of their life history. And, to add to the complexity, there are many species of rust fungi which are subdivided into races which cannot be distinguished morphologically, but which are physiologically distinct.

The well known species *Puccinia graminis* exhibits all the complexities just mentioned. It consists of several distinct races, all structurally alike, but showing peculiarities in the type of grasses on which they will grow. One will grow on rye or barley but not on oat; another will grow on wheat, barley, rye and oat, but not on other grasses, and the other races exhibit similar peculiarities. All the races of *Puccinia graminis* are heteroecious, with the aecidium stage growing on the barberry. In the spring the wheat rust makes its appearance as rust colored spots or streaks on the stalks and leaves of the immature plant. The colored spots are due to the presence of a layer of countless numbers of minute brown spores, the uredospores of the summer fruiting form. The uredospores are carried by the wind or insects to other wheat plants where they germinate, sending mycelium into the tissue, and developing the rust spots on the leaves and stalks. This phase of the life cycle, which requires about two weeks for its completion, is repeated throughout the summer, but toward the end of the summer the uredospores are replaced by the winter resting spores, called teleutospores, which are larger, thicker walled and darker in color. The teleutospores remain inactive on the straw until spring, when they germinate on manure

heaps or on moist ground and produce minute sporidia, which are conveyed by the wind to the alternate host, the barberry. In due time the fungus known as *Aecidium berberidis* appears on the barberry leaves in the form of small cluster-cups, each of which is filled with chains of orange-colored aecidiospores. Infection of the young wheat plants follows the scattering of the aecidiospores. The rust spots of uredospores develop, and the life cycle is completed.

The life history of the wheat rust is even more complex, however, than the outline just given displays. On the barberry, in addition to the fertile aecidiospores, there is produced also another kind of spore—the spermatia—which do not germinate. They are generally regarded as abortive male cells. Further, it is not only the gross structure which is involved in the various changes occurring in the life cycle of the Uredinae, but also the internal structure of the cells. The aecidiospores, the uredospore bearing mycelium, the uredospores and the young teleutospores all contain a pair of nuclei. Before the teleutospore reaches maturity the nuclei fuse, and the uninucleate condition continues through the sporidia, the aecidium-bearing mycelium and the spermatia.

Although the normal and complete development of the wheat rust is as outlined above, the cycle does not always follow the complete course. In Australia the barberry is an imported plant, and of rare occurrence, yet wheat rust is very abundant. The uredospore phase of the cycle, which is interrupted in temperate climates by the intervention of cold weather, continues indefinitely in the warmer Australian climate.

The case of life cycles in algae is of particular interest to bacteriologists on account of the close relationship between the Cyanophyceae, or blue-green algae and the Schizomycetes. Although there is a marked contrast in the method of nutrition of these two groups, their morphological affinity is acknowledged.

Of the thousands of species of algae which have been described, relatively few have been traced through their life cycle from spore to spore, owing to the aquatic habit of most of the species and the minute size of many of them. Consequently there is uncer-

tainty about life histories. Most algologists have accepted pleomorphism, but they differ in opinion concerning the extent of pleomorphism. Whether all algae are pleomorphic, and how many phases one species may pass through, are the questions on which the algologists disagree. Some claim that the simpler forms of algae pass through a number of distinct phases in their life history. Others admit that metamorphoses occur, but believe that the phases of a given organism are few in number. One investigator reports that *Protoderma viride* passes through a series of changes so varied that at different times it presents the characters of twelve different genera. Another investigator does not find so great a pleomorphism, but reports that *Raphidium* passes through phases represented by five different genera. A third investigator confirms the observation that *Raphidium* undergoes a metamorphosis by which it is converted into an organism with the characteristics of another genus, but was unable to observe further metamorphoses.

The striking contrast between the gradual changes which occur in the individual mammal, and the abrupt changes in morphology, accompanying changes in mode of life, which occur in lower forms corresponds with differences in exposure to environmental changes. In mammals, the life of the individual begins with an ovic cell, which develops during embryonic life in a carefully controlled environment with constant internal temperature, moisture and food conditions insuring a definite course of development. Throughout the life of the individual there is an interchangeability of body fluids among the various organs; and there is a dependence of form, rate and extent of growth and maturity upon internal secretions. The interdependence of one organ upon another secures a balance and continuity of development along prescribed lines with protection against disturbing influences of environment, which is in contradistinction to the conditions found in lower forms of life. In lower forms, as, for example, in insects, the mechanisms for maintaining constant conditions are less perfected than in mammals, but this defect is counterbalanced by a capacity for adjustment to variations in environment.

External influences operate most vigorously upon the one-

celled organisms, for in them the facility for an interchange of fluids is deficient or lacking. When one-celled organisms grow in masses, however, the individual cells influence and protect one another, as evidenced by the fairly common observation that bacteria sparsely seeded in a given medium may fail to grow, although they will grow in that particular medium if the seeding is more abundant. Whatever interchange of fluids there may be between isolated individuals of singled celled organisms is dependent on flux through the medium in which they are growing. Therefore the more complete the isolation of an individual, the more exposed it is to environmental factors.

In the examples cited of metamorphoses in two widely divergent forms of life, insects, on the one hand, and algae and fungi on the other, changes which occur in life cycles in response to environmental changes generally follow definite lines. With a given set of conditions the same form of the organism appears as appeared under those conditions in preceding years; and irregularities in environmental conditions result in irregularities in the life cycle.

Life cycles in bacteria should be expected, in accordance with the general biologic law that in the lower simple forms of life there is a capacity for irregular and sudden metamorphoses to adjust the organism to the vagaries of environment with special forms for special circumstances; and notable irregularities in the cycle should be expected in bacteria, because the influences of environment operate on exposed single cells.

If bacterial organisms follow life cycles of a complexity comparable to those of fungi, it will be an extremely difficult problem to demonstrate the changes. Bacteriologists will not quail, however, because they are confronted with difficulties. Every evidence of change in bacterial cultures should be examined with a mind ready to consider the possibilities of pleomorphism as well as to consider the possibilities of contamination. Let us therefore survey briefly the evidence of pleomorphosis and pleobiosis in bacterial organisms.

In his recent monograph on microbic dissociation, Hadley has ably considered the instability of bacterial species in such char-

acteristics as the kind of colony, antigenic power, agglutinability, and virulence. The changes which Hadley considered are, in general, changes which would implicate variety or species identification, according to present systems of classification. This discussion will be limited to the more profound changes of morphology and biologic behavior—changes of a magnitude comparable with the change from the uninucleate uredospore phase of the wheat rust, which can grow only upon grasses, to the binucleate aecidiospore phase which can grow only on the barberry. Changes of this order would move the organism from one genus or family to another, according to systems of bacterial classification now in use.

Metamorphosis is the term used by zoologists to describe the sudden changes in morphology which occur in life cycles. It is also used to describe sudden changes of morphology in the life cycles of the algae. The term has not been used by many bacteriologists, although it appears to be appropriate to describe bacterial changes which are comparable with the changes which occur in the animal kingdom and among algae.

As applied by zoologists to certain species, metamorphosis designates the changes through which a single individual organism passes, as for example, in butterflies. In other species, as in the trematodes, the cycle is spread over several generations, and the term metamorphosis is applied to the transformations which occur in the complicated development through the sporocyst, redia and cercaria stages, each stage representing a new generation, or series of generations descended from the adult trematode, and not three distinct genera, as was once supposed. The use of the term metamorphosis in bacteriology would be more comparable with the latter example.

Unfortunately the term mutation is used commonly by bacteriologists to describe the changes which occur in life cycles. The term mutation is generally applied by biologists to irreversible changes. Therefore it can not be correctly applied to the changes which occur in life cycles. It is probable that phylogenetic mutations giving rise to new taxonomic units occur in bacteria as in other forms of life, but until the changes that occur in

the ontogenetic life histories of bacterial organisms are known, it will be impossible to recognize an unusual change which could be correctly designated as a mutation. It is true that many investigators have reported that their mutated strains breed true indefinitely. In every such case, however, the unanswerable question arises whether the unknown conditions which might induce a reversion to the original type have been supplied.

Since the early days of the science, the theory of life cycles in bacteria has had its advocates. Billroth, Nägeli, Almquist, Adami, Hort and others recognized pleomorphism in bacteria. They were unable to assemble sufficient convincing data, however, to combat the prevailing monomorphic theory.

Löhnis reviewed the evidence of life cycles in bacteria which appeared in the literature up to 1918, and his monograph includes an extensive bibliography. In 1925 Enderlein continued the argument and presented further evidence in favor of life cycles.

New evidence of metamorphoses in bacteria appears from time to time and its value should be appraised. Let us examine a few of the most convincing observations which have been reported.

In 1909 Bredemann reported that it is possible to transform *Bacillus amylobacter*, an anaerobic spore-bearing bacillus, which vigorously ferments carbohydrates, into an aerobic, non-spore-bearing and non-fermenting coccus. He stated that he could induce this transformation in all his strains, but he was unable to revert the coccus form into the typical anaerobic bacillus. He was convinced of the purity of his cultures. In 1927 Cunningham and Jenkins confirmed Bredemann's observations. They studied six strains of *B. amylobacter* from as many different sources, and obtained coccus cultures from two of them by cultivation under incomplete anaerobiosis. Careful precautions were taken to ascertain the purity of the cultures. Fourteen strains of coccus cultures derived from *B. amylobacter* were studied. Two of them produced pigment, and agreed with the descriptions of *Micrococcus aurantiacus*. The remainder of the coccus cultures produced no pigment, and agreed with the descriptions of *Micrococcus candicans*.

By cultivating in sugar agar pure strains of a bifurcated, non-motile, non-spore-bearing anaerobic organism, Noguchi was able to induce its transformation into a motile, spore-bearing, aerobic bacillus; and by gradual training to the anaerobic life he was able to induce a reversion to the original phase of the organism. Howe and Hatch confirmed Noguchi's observations. They succeeded in producing a sporulating stage from the bifurcating form, and in transforming the spore-bearing to a non-spore-bearing stage.

While investigating the etiology of malignant granulomata, Negri observed remarkable pleomorphism in his cultures. In order to ascertain whether the transformations were due to contaminations, he obtained single cell cultures of the organism. With all precautions against contamination, he was still able to effect the transformations. He used the term metamorphosis to describe the changes which he observed. Various coccoid and diphtheroid forms could be induced, dependent on the nature of the medium on which the organism was grown. Very soon after Negri's report, Löhnis and Smith's *Life Cycles of the Bacteria* was published, in 1916. These workers studied the pleomorphism of Azotobacter and found that it undergoes a complicated life cycle. They then studied various other bacteria, and found that every species studied exhibited most of the forms found in Azotobacter. Löhnis and Smith acknowledged that their observations were essentially identical with those of Negri. Kellermann and Scales then took up the study of life cycles in bacteria. They studied twelve strains of *Bacillus coli* (*Escherichia coli*) from widely differing sources, and they observed in these cultures all the forms, excepting the spore-bearing, described by Löhnis and Smith.

In 1923 Löhnis and Smith reported further studies on 30 strains of Azotobacter. They found seven different cell forms, each with its own peculiar physiologic behavior. The various phases represented five different genera—*Micrococcus, Bacterium, Pseudomonas, Bacillus* and *Mycobacterium.*

In 1919 Mellon described a fuso-spirillary organism which grew as branching filaments, cocci or rods. There was also a filtrable phase. In a more recent publication Mellon emphasized the virulence of the filtrable or gonidial phase of the organism.

Mellon and Yost showed that "*Bacillus alkaligenes*"—known as a rod form—may grow in the human body as a Gram-positive diplococcus and that it can be cultivated as such on artificial media for years. It may also grow as a streptothrix, and there is another phase of mixed morphology.

Tunnicliff and Jackson cultivated from a tonsillar granule a pleomorphic organism which, in its most frequently observed and stable form, was a short bacillus. During the course of its life cycle it appeared also as large and small coccoid forms, and as straight and wavy filaments. The organism multiplied by fission, by budding, and by the production of gonidia.

In my study of the etiology of epidemic encephalitis I have made some observations which add to the evidence of metamorphoses in bacteria. I found that *Bacillus subtilis* and certain other common saprophytic bacteria are virulent when inoculated into the brains of rabbits. Some strains of *B. subtilis* are so virulent that they kill the rabbits within two or three hours if a small quantity of broth culture is inoculated into the brain. Other strains are less virulent, and, occasionally, one may be found which will produce symptoms from which the rabbit will recover within a few days. After a few weeks or a few months the recovered rabbit may develop symptoms of encephalitis and die after a brief illness. The brain of such a rabbit contains a virus which is indistinguishable from the well known so-called herpetic and encephalitic viruses. From the brains of rabbits which die following inoculation of this virus, and from human encephalitic material both *B. subtilis* and streptococci may be cultivated irregularly in chopped meat medium. In the first and second culture generations the bacterial organism is unstable, and metamorphosis may be observed. After a few transfers the form of the organism becomes stabilized, and will remain pure so long as it is maintained in culture media with occasional transfers. I have seen the transformation of rod to streptococcus so many times that I am forced to the conclusion that *B. subtilis* with its endospores is the resistant resting phase of an organism with a complex life cycle, certain phases of which are parasitic in mammals. The details of these experiments and observations will be published elsewhere.

This is not a proper occasion to continue the citation of instances of metamorphoses in bacteria. The extensive bibliographies in the monographs of Löhnis, Enderlein and Hadley are available to those who want to investigate the subject. The examples of metamorphosis already cited are sufficient to establish beyond a reasonable doubt that metamorphosis does occur in bacterial organisms. The questions now open to consideration are; whether all bacteria undergo metamorphoses; and through how many phases a single species may pass.

There are a number of striking coincidences of particular bacterial forms commonly found in certain diseases although the evidence indicates that they are not the primary cause of the disease with which they are associated. Thus, streptococci are associated with epidemic encephalitis, although some unknown virus is generally regarded as the cause of the disease; similarly, streptococci and "globoid bodies" are associated with poliomyelitis, a disease caused by a filtrable virus; and streptococci are also associated with measles, another disease in which an unknown filtrable virus is generally regarded as the cause; "Proteus X19" is associated with typhus, although apparently it does not possess pathogenic power; *Salmonella suipestifer* is associated with hog cholera, a disease caused by a filtrable virus; and the so-called *Bacillus bronchisepticus* is associated with the pneumonic type of dog distemper, although the virus of this disease, also, is filtrable. In the case of some of these conditions, the possibility that the associated bacteria may be a phase in the life cycle of the causal organism has already received consideration. The study of these diseases offers a field which may serve as the proving-ground for the pleomorphic theory.

Bacteriologists need not feel chagrinned if they have to admit that they cannot follow the life cycles of bacteria; and that forms they have considered as different genera are but stages in the life cycle of one species. There is solace in the thought that the difficulty in following a life history increases in inverse proportion to the size of the organism. Yet the zoologists once thought the larval stage of the lamprey was a separate genus, which they called *Ammocoetes*. The zoologists cannot arrive at a satisfactory

classification of the Hydromedusae because they do not know their complete life cycles; they do not in all cases know the polyp corresponding to a given medusa, nor the medusa that arises from a given polyp. The helminthologists cannot find the larval stage of certain cestodes parasitic in sheep; to this day the larval stage of the human tapeworm, *Taenia solium*, is called *Cysticercus cellulosae*. The mycologists readily admit their inability to discover the complete life cycle of a great number of species of fungi; and the algologists are in the same confused state of mind in regard to life cycles as are bacteriologists.

There is a story told of a New England philosopher who while driving through the country came upon a curious, low, rambling house in building. He went up to the house and inquired of a carpenter "Who is the architect of this building?" The carpenter replied, "We have had none yet, but we are going to send to Boston before long to get some one to come and put in the architecture."

The science of bacteriology is in some respects a rambling structure of incoordinated knowledge, and the time has come when it is at least as important to audit and correlate existing knowledge as it is to learn new isolated facts. A vast amount of information has been accumulated about this and that and the other form as they occur in particular habitats, but we are only beginning to appreciate the possibilities of the revelations which may await the study of life cycles in bacteria. The theory recently promulgated by Hadley that bacteriophage is a phase in the life cycle of bacteria adds a new conception involving practical applications.

A knowledge of life cycles would presumably influence industrial bacteriology the least of the several branches of the science, for the conditions maintained in industrial processes are more or less under control, and as a result the useful or harmful bacterial forms which are encountered are limited to those which can multiply under the given conditions. On the other hand, the significance of life cycles in bacterial organisms to soil science, phytopathology, medical bacteriology, immunology and epidemiology can only be imagined, for in all his relationships with bacteria in their natural habitats man is compelled to deal with all of their potentialities.

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A COLORIMETRIC METHOD FOR THE DETERMINATION OF BUTYRIC ACID[1]

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The determination of the different kinds of volatile acid produced by bacteria is a problem that presents many difficulties. Duclaux's method is the recognized procedure, but unfortunately this method takes a great deal of time, requires very carefully standardized apparatus, and considerable skill and experience on the part of the operator. When large numbers of cultures have to be examined for the purpose of sorting out those which produce a particular acid, e.g. butyric, it is practically impossible to use the Duclaux method. A more rapid method even though it does not possess the accuracy of the Duclaux procedure is decidedly helpful.

A qualitative colorimetric test for butyric acid in a mixture of volatile acid was reported by Agulhon in 1913 and slightly modified by Dyer (1917). By employing more suitable conditions for applying this test and by introducing a set of standard color tubes we have made the method quantitative.

EXPERIMENTAL

The general principle of Agulhon's test is to convert butyric acid into its copper salt and to extract this with ether. The copper butyrate imparts a blue color to the ether layer. The degree of color varies with the concentration of butyrate, the acidity of the solution and the quantity of copper salt used.

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With ether as solvent, precipitates frequently form and invalidate the test. We have found chloroform to be a more satisfactory solvent than ether, and by choosing the proper conditions, have overcome the formation of precipitates. After a large number of trials the following method has been developed.

Reagents

1. Copper chloride—hydrochloric acid reagent.

Dissolve 85.26 grams of $CuCl_2 \cdot 2H_2O$ in 1000 cc. of 1 N HCl.

TABLE 1

Composition of acetate-butyrate mixtures for preparation of standard color tubes

TUBE NUMBER	0.5 N SODIUM ACETATE	0.5 N SODIUM BUTYRATE	PERCENTAGE COMPOSITION	
			Acetic acid	Butyric acid
	cc.	cc.		
1	2.55	0.45	85	15
2	2.40	0.60	80	20
3	2.25	0.75	75	25
4	2.10	0.90	70	30
5	1.95	1.05	65	35
6	1.80	1.20	60	40
7	1.65	1.35	55	45
8	1.50	1.50	50	50
9	1.35	1.65	45	55
10	1.20	1.80	40	60
11	1.05	1.95	35	65
12	0.90	2.10	30	70
13	0.60	2.40	20	80
14	0.30	2.70	10	90

2. Chloroform—C. P.

3. Standard color tubes.

Make up half normal solutions of sodium acetate and sodium butyrate as follows:

Dilute 3 grams of glacial acetic acid with 20 cc. of water and neutralize to phenolphthalein with 1 N NaOH. Dilute the resulting sodium acetate solution to twice the volume of the 1 N NaOH used. This gives a solution of exactly 0.5 N strength.

Redistill butyric acid (Eastman Kodak Company) and collect

the fraction boiling between 161 to 162°C. Dilute 4.4 grams of this distillate with water and treat in the same manner as above to give a 0.5 N strength solution of sodium butyrate.

Mixtures of these two solutions are made as indicated in table 1. The combinations are made in a separatory funnel and to each combination 0.4 cc. of $CuCl_2$-HCl reagent are added and the mixture shaken. Five cubic centimeters of chloroform are then added and shaking continued until a maximum color develops in the chloroform layer (twenty to thirty seconds). After the chloroform layer has settled it is drawn off into test tubes of uniform diameter and closed with a well-fitting cork of good quality. The cork is cut off level with the top of the tube and covered with a 10 per cent solution of gelatin. The level of the liquid in the tube should be marked with a file so that if evaporation does take place it may be detected.

Preparation of sample for analysis

If the volatile acids are contained in cultures the following procedure is used for their removal.

To 50 cc. of the culture (or a quantity that will give at least 3.0 cc. of 0.5 N volatile acid) add 20 grams of $NaH_2PO_4 \cdot H_2O$, 15 cc. of 85 per cent H_3PO_4 and distill off the volatile acids. With a blue pencil draw a line on the distilling flask at 50 cc. volume and keep within 10 to 15 cc. of this volume during the distillation by adding water from a dropping funnel at about the same rate as the liquid distills.

Collect 200 cc. of distillate which will contain practically all of the volatile acid (Fred et al., 1919). Titrate the distillate with 0.5 N or other standard NaOH to the end point of phenolphthalein and evaporate to such a volume as will be slightly stronger than a 0.5 N solution. If the solution shows any traces of pink color, neutralize with HCl and dilute to an exactly 0.5 N solution.

To exactly 3 cc. of the neutral solution add 0.4 cc. of $CuCl_2$-HCl reagent and shake. Then add 5 cc. of chloroform and shake until a maximum coloration develops in the chloroform layer. After settling, the chloroform layer is drawn off into a test tube of the same diameter as the standard tubes and matched against the standards.

Source of light for comparing tubes

Comparison of the unknown with the standard tubes is best accomplished by the use of electric light. The most suitable source of light was found to be a white frosted bulb placed in a metal container. A small opening, 1 by 1 inch, was cut in this container and the opening covered with a piece of onion skin typewriter paper. The unknown and standard are held close to this opening and compared. The comparison is repeated with various standards until the best possible match is obtained. If the unknown falls between two of the standards, the value of the unknown is interpolated.

If the unknown solution contains more than 70 per cent of the total acid in the form of butyric, it is better to use 0.25 N solution of both standards and unknown, and to reduce the quantity of $CuCl_2$-HCl reagent to 0.2 cc. Owing to the intensity of color produced it is difficult to match the higher concentrations of butyric acid. If the unknown contains less than 20 per cent of the acid in the form of butyric, the method can be made more sensitive by using 1 N solutions of both standards and unknown and by increasing the quantity of $CuCl_2$-HCl reagent to 0.8 cc. In any case the smallest quantity of butyric acid that can be detected is about 20 mgm.

Factors influencing color

Concentration of $CuCl_2$ and HCl. The color test is presumably based upon the following reaction:

$$2\ C_3H_7CO_2Na + CuCl_2 \rightleftharpoons (C_3H_7CO_2)_2\ Cu + 2\ NaCl$$

$(C_3H_7CO_2)_2Cu$ is soluble in certain organic solvents and imparts a blue color to the solvent. Secondary reactions seem to occur due perhaps to the hydrolysis of the $(C_3H_7CO_2)_2Cu$ with the formation of basic insoluble compounds. To prevent the formation of these precipitates and to insure the maximum of color a certain amount of free acid is necessary.

In the course of developing the method which has just been described a number of tests were conducted to ascertain the most

suitable conditions for the procedure. Table 2 shows the change in color and effect on precipitate formation by varying the quantities of $CuCl_2$ and HCl. All the tubes contained the same quantity of volatile acid, 3 cc. of 0.5 N. In the first series this

TABLE 2

Effect of acidity and concentration of copper chloride on color intensity in chloroform layer

TUBE NUMBER*	1 N HCl	2 N $CuCl_2$	APPARENT CONTENT OF BUTYRIC ACID		FORMATION OF PRECIPITATE
			Series 1	Series 2	
	cc.	cc.	per cent	per cent	
1	0.30	0.15	40	80	None in 3 months
2	0.45	0.15	40	80	None in 3 months
3	0.60	0.15	34	73	None in 3 months
4	0.40	0.10	31	60	None in 3 months
5	0.40	0.20	40	87	None in 3 months
6	0.40	0.30	47	100	After 27 days
7	0.40	0.50	55	100+	After 12 hours
8	0.60	0.20	38	77	None in 3 months
9	0.60	0.30	43	100	None in 3 months
10	0.60	0.50	45	100+	After 48 hours
11	0.80	0.20	33	73	None in 3 months
12	0.80	0.40	33	73	None in 3 months
13	0.80	0.60	33	73	After 24 hours
14	0.80	0.50	33	73	None in 3 months
15	0.80	0.50	33	73−	After 3 days
16	0.80	0.80	33	73	After 12 hours

* Both standards and tubes under test contained 3 cc. of 0.5 N acid. Of this acid 40 per cent was butyric in Series 1 and 80 per cent in Series 2. The standards contained 0.4 cc. of 1 N HCl and 0.15 cc. of 2 N $CuCl_2$; the tubes under test contained the quantities indicated in columns 2 and 3.

consisted of 60 per cent acetic and 40 per cent butyric acid; in the second series the proportion was 20 per cent acetic and 80 per cent butyric acid. After adding the HCl and $CuCl_2$, water was added to make a constant volume of 4 cc. and the solution was then shaken with 3 cc. of chloroform. Comparison of the color pro-

duced was made with standards similarly prepared but containing 0.4 cc. 1 N HCl and 0.15 cc. 2 N $CuCl_2$.

The data in table 2 show that with a fixed quantity of $CuCl_2$ the color in general decreased as the HCl increased. If nos. 1 and 3, and nos. 5 and 11 are compared, it is seen that doubling the quantity of HCl decreased the apparent percentage of butyric acid from 6 to 14 per cent.

If the HCl is kept low and constant, the color is increased as the $CuCl_2$ is increased—see nos. 4 to 10 inclusive. The effect is more pronounced with the higher percentages of butyric acid than with the lower. A limit is soon reached, however, as the formation of a precipitate sets in, and makes comparison impossible. There is no advantage in increasing the quantity of $CuCl_2$ above a certain point, since the deep blue color developed with the higher percentages of butyric acid becomes too intense for comparison.

Many other combinations of HCl and $CuCl_2$ were tried and on the basis of these tests, the combination in no. 5 was adopted as having the most desirable features over the whole range of acetic and butyric acid mixtures. This is the combination used in the standard color tubes and unknown solutions.

In the preliminary tests separate solutions of 2 N $CuCl_2$ and 1 N HCl were used. This did not prove satisfactory as the aqueous solution of 2 N $CuCl_2$ developed a yellow precipitate, presumably an oxide of copper, upon standing a few weeks. This reduced the concentration of the solution rendering it useless for the test. To avoid this effect, the $CuCl_2$ and HCl were combined and used as one reagent. No precipitate resulted with this combination and a saving of time in making the test was effected.

Effect of other acids. The presence of propionic acid produces high results, inasmuch as copper propionate is partially soluble in chloroform and produces a blue color. Several solvents were used in an attempt to eliminate this factor but to no avail. Also the Haberland (1899) method for the separation of propionic acid was tried but failed to give satisfactory results. Fortunately propionic acid is usually not formed by the bacteria that produce butyric acid. In pure culture work interference by propionic acid is, therefore, not a serious matter, but in mixed cultures the method becomes inapplicable.

Small amounts of formic acid, up to 25 per cent of the total, do not affect the determination, but larger quantities give low results. In cultures which produce butyric acid it is probable that formic acid to the extent of 25 per cent of the total would never be formed.

Use of other metallic salts and solvents. Cupric sulphate, cupric chloride, ferric chloride and cadmium chloride were tried in an attempt to find a suitable salt which would react with sodium butyrate giving a characteristic color to the solvent layer. Of these cupric chloride was found to produce the best results.

The following solvents were tested to find a suitable medium for color comparisons: carbon tetrachloride, carbon disulphide, petroleum ether, ethyl ether, chloroform, xylene, benzene, benzaldehyde, octyl alcohol, amyl alcohol, amyl acetate and ethyl acetate. A blue color developed in all cases except with carbon tetrachloride and carbon disulphide. Ether and chloroform gave the maximum and about the same intensity of color. Ether was found to be objectionable because of the tendency to form emulsions and the slowness with which the two layers separated. Moreover, precipitates and a fading of color resulted even when the ether layer was separated from the mixture. The chloroform layer, on the other hand, separated almost immediately and showed less tendency to form precipitates. Chloroform possesses another advantage in that it does not evaporate as readily as ether and thus permits the preparation of permanent standards.

Comparison with the Duclaux method

The colorimetric method was checked a number of times with mixtures of pure acetic and butyric acids and found to give accurate results. It was then tried out on cultures of butyric acid-forming bacteria. Acids from two types of such microörganisms were tested. One group is made up of the acetone-butyl alcohol microörganism, represented by cultures 70 and 105 and the other group consists of the non-butyl alcohol-producing bacteria and includes a number of widely differing bacteria which are designated in the table by nos. 2, 9, 21, 22,

24, 25, 31, 32, 36. In all seventeen cultures were used and the results by the two methods are given in table 3. The difference between the two methods ranged from +6.0 per cent to −6.5 per cent. This degree of accuracy is probably sufficient for most

TABLE 3

Comparison of the colorimetric and Duclaux methods for the determination of butyric acid in bacterial cultures

CULTURE NUMBER	BUTYRIC ACID BY		DIFFERENCE
	Colorimetric method	Duclaux method	
True butyric acid bacteria			
	per cent	*per cent*	*per cent*
2	27.0	28.4	−1.4
9	49.0	54.1	−5.1
21	66.0	65.4	+0.6
22	66.0	70.3	−4.3
22	77.0	73.1	+3.9
24	73.0	76.8	−3.8
24	73.0	74.0	−1.0
25	80.0	79.5	+0.5
25	60.0	66.5	−6.5
31	57.0	59.8	−2.8
32	73.0	71.2	+1.8
36	60.0	65.2	−5.2
36	58.0	59.0	−1.0
36	80.0	74.0	+6.0
Acetone butyl-alcohol bacteria			
70	47.0	43.3	+3.7
105	56.0	54.5	+1.5
105	48.0	44.6	+3.4

fermentation work and in the sorting out of large numbers of butyric acid-producing bacteria should save a great deal of time.

SUMMARY

A colorimetric method for the estimation of butyric acid in the presence of acetic and formic acids is described. The solution to be tested is made to a definite strength and is then treated

with a $CuCl_2$-HCl reagent and the mixture shaken with chloroform. Known mixtures of butyric and acetic acids are treated in exactly the same manner and are used as standards for comparison with the unknown. By carefully standardizing the procedure good checks were obtained with the Duclaux method on 17 cultures producing butyric acid.

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BACTERIAL ANTAGONISM WITH SPECIAL REFERENCE TO THE EFFECT OF PSEUDOMONAS FLUORESCENS ON SPORE FORMING BACTERIA OF SOILS

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Bacterial antagonism has been extensively studied in the past by many investigators. The voluminous literature of the subject is fully reviewed in Kolle and Wassermann's *Handbuch* and requires but brief consideration here.

Pseudomonas fluorescens, *Ps. putida* and *Ps. pyocyanea* are frequently mentioned in the literature as organisms which exert an inhibitory or bactericidal action on other species of bacteria. There are conflicting reports, however, as to the organisms inhibited and concerning the nature and cause of antagonism. As early as 1887, Garre studied the effect of *Ps. putida* on several species of bacteria. He found that *Staph. aureus*, *B. typhosus*, and *B. mucosus-capsulatus* were inhibited while *B. anthracis* and the vibrio of Finkler and Prior grew normally. These results were not in complete accord with those of Lewek (1889) who found that in association with *Ps. putida*, *B. anthracis* and *Staph. aureus* were both killed, *Staph. albus* grew feebly, *V. cholera* was retarded while *B. typhosus* and *B. coli* grew vigorously. *Ps. fluorescens* had no effect on *B. typhosus*, *Staph. aureus* or *V. cholera* but was strongly antagonistic for *B. anthracis*. Olitsky (1891) determined that *B. typhosus*, *B. anthracis*, *V. cholera*, *B. prodigiosus* and *Staph. aureus* are markedly inhibited by *Ps. fluorescens* These results are at variance with those of Garre as to the effect on *Staph. aureus* and *B. typhosus*.

The survival of *B. typhosus* in sewage was studied by Laws and Andrewes (1891) who found that the period was shortened in the presence of *Ps. fluorescens*. Horrocks (1901) was unable to

recover *B. typhosus* after it had been incubated for seven days with *Ps. fluorescens* in sterilized sewage. Frost (1904) found the metabolic products of both *Ps. fluorescens* and *Ps. putida* to be not only inhibitory but also bactericidal for *B. typhosus*.

Other investigators, not concerned primarily with the question of antagonism, have cultivated *Ps. fluorescens* in mixed association with other species. Luxwolda (1911) studied the growth of *Ps. fluorescens* and *Strep. lacticus* separately and in mixed association in milk at different temperatures. At temperatures of 20, 15, 13 and 10°C., both species appeared to be benefited by the association.

Conn and Bright (1919) inoculated sterilized manured soil with a mixture of *B. cereus* and *Ps. fluorescens* cells and made subsequent quantitative analyses for a period of fifteen days to determine the relative rate of multiplication. They failed to obtain colonies of *B. cereus* in the dilutions plated while *Ps. fluorescens* had multiplied enormously. The same result was obtained when the ratio of cells in the inoculum was greatly in favor of *B. cereus*. Similar results were obtained in unsterilized manured soil. *B cereus* failed to increase appreciably under the conditions of these experiments.

Waksman and Lomanitz (1924) cultured *B. cereus* and *Ps. fluorescens* separately and in mixed association in flasks of casein solution. They found that *B. cereus* was very active in this medium while *Ps. fluorescens* failed to attack the substance vigorously when growing alone. In the flask inoculated with the two species, it was found that *B. cereus* decomposed the casein, producing amino acids which were further decomposed by *Ps. fluorescens*, as soon as formed, with the production of ammonia. The exact quantitative bacterial analysis of the mixed culture is not given in the report of these authors. They state, however, "the number of *Ps. fluorescens* cells greatly exceeded those of *B. cereus;* the culture looked and smelled like a typical *Ps. fluorescens* culture."

The present investigation was undertaken for the purpose of determining more fully the effect of *Ps. fluorescens* on some species of spore-forming soil bacteria and what influence if any is exerted

on their development in soil by the bacterio-toxic substance. Additional species were included in the experiments in order to determine relative susceptibility.

EXPERIMENTAL METHODS

Various methods have been used for the study of bacterial antagonism. The method of Garre (l.c.) has been employed extensively by others, either in its original form or with slight modifications. Agar or gelatin is poured into Petri dishes, allowed to harden and inoculated on the surface with the two species placed alternately either parallel or radiating from the center.

This method has some value but it is generally less satisfactory than other methods. Very striking results are obtained when a spreading form such as *B. mycoides* is grown in association with *Ps. fluorescens*. As the filamentous rhizoid-like growth approaches the inhibitory zone it halts abruptly and is unable to make a further advance. The growth of *B. mycoides* on the side adjacent to the inhibitor contrasts sharply with that on the opposite side. This method affords the advantage of permitting either simultaneous inoculations or a period of time for the growth of the inhibitor.

A more useful plate method of cultivating two species in mixed association is the seeded plate with surface inoculation. The agar, seeded with one of the pair, is poured into a Petri dish and inoculated on the surface with the other.

The results are striking when such a strongly inhibiting organism as *Ps. fluorescens* is grown on the surface of agar seeded with an inhibited species which has a slow normal rate of development. The extent of the inhibited zone depends on several factors, of which the composition and consistency of the agar, its depth in the dish, amount of inoculum, susceptibility of the inhibited species and the normal growth rate of the two species appear to be the more important.

When agar is seeded, not too heavily, with spores from an old culture of *B. mycoides* and inoculated on the surface with *Ps. fluorescens* the zone of inhibition reaches a width of 2 or 3 cm.

(fig. 2). In plates seeded with young cultures containing actively growing cells the results are much less marked. In such slow growing species as *Sarcina lutea* and *Lactobacillus flavus* the zone in which colonies fail to develop extends almost the full width of the dish. Time is required for the accumulation of metabolic

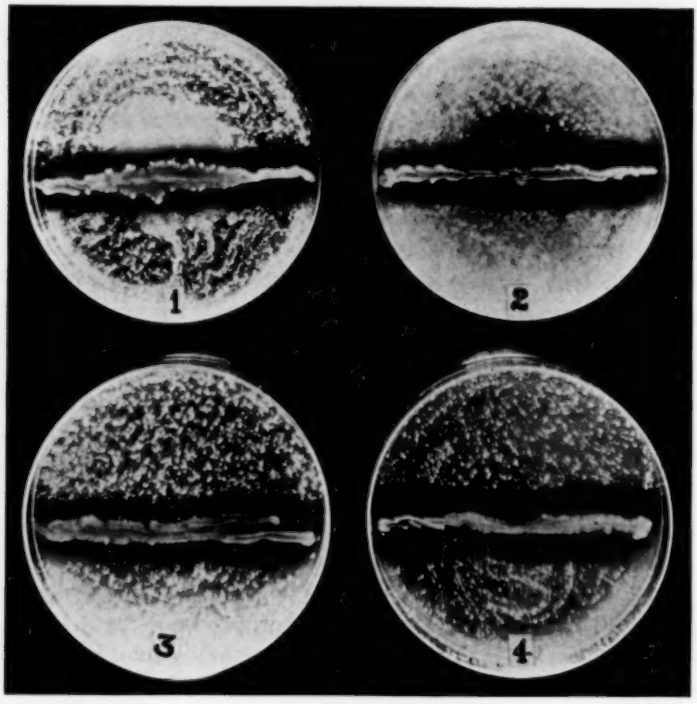

FIG. 1. B SUBTILIS
FIG. 2. B. MYCOIDES
FIG. 3. B VULGATUS
FIG. 4. B. CEREUS

products in sufficient concentration to check development of the colonies. With such species as *B. coli*, *Serratia marcescens* and others the colonies are already developed before this point is reached.

The seeded plate method is useful for preliminary reconnaissance, but its application to the study of bacterial antagonism is

limited and the results, when negative, are very often misleading. For the more precise study of antagonism other methods are preferable. The collodion sack method of Frost gives reliable results but it is difficult and time consuming when many tests are to be made.

In the present investigation, the seeded plate method with surface inoculation was used for preliminary experiments. In order to determine more definitely the degree of inhibition, old cultures of *Ps. fluorescens* were sterilized and added to nutrient agar in various proportions. The agar base was made of proper strength to contain 0.5 per cent peptone, 0.3 per cent beef extract, and 2.0 per cent agar after the addition of the sterilized old culture. The final reaction was adjusted to pH 7.2. The finished agar was poured into Petri dishes and inoculated on the surface with the organisms to be tested.

A suitable culture medium for the production of toxin was determined by experiment. It was considered desirable to use a synthetic medium if possible. Media prepared according to the formulae of Uschinsky (1893), Jordan (1899), Braun and Cahn-Bronner (1921) and Fraenkel (1894), as well as a variety of non-synthetic media were tested. The latter included casein and blood digest; peptone; peptone plus beef extract; peptone plus gelatin, and peptone, beef extract plus glucose. The toxic substance was developed in all media but was found to be more abundant in Uschinsky's medium. Peptone beef extract agar was used for the seeded plate method and either Uschinsky solution or agar for all other experiments.

The strain of *Ps. fluorescens* was isolated from water. It gave all the typical reactions for the species and produced abundant pigment in all suitable media. The test organisms were principally stock cultures carried in this laboratory but some were freshly isolated from original sources.

EFFECT OF PSEUDOMONAS METABOLITES ON OTHER SPECIES OF BACTERIA AND FUNGI

Preliminary experiments, in which the seeded plate method was employed, showed that some species are strongly inhibited while

TABLE 1

NAME OF ORGANISM	PER CENT OF AGED MEDIUM IN THE AGAR (PLUS SIGN DENOTES COMPLETE INHIBITION)									
	0.5	1.0	2.5	5.0	10	15	20	30	40	50
B. cereus	−	−	+							
B. mycoides	−	−	+							
B. anthracis	−	+								
B. sp. Number 27	−	−	+							
B. Sp. Number 30	−	−	+							
B. vulgatus	−	−	+							
B. subtilis	−	−	+							
B. megatherium	−	+								
Rhodococcus cinnebareus	−	+								
Rhodococcus roseus	−	−	+							
Micrococcus flavus	−	−	−	+						
Neisseria catarrhalis	−	−	−	+						
Ps. aeruginosa	−	−	−	−	−	−	−	−	−	−
Ps. fluorescens	−	−	−	−	−	−	−	−	−	−
Sarcina lutea	−	−	−	+						
Sarcina aurantiaca	−	−	+							
Serratia marcescens	−	−	−	−	−	+				
Staph. albus	−	−	+							
Staph. aureus	−	−	−	+						
Staph. citreus	−	−	+							
Encapsulatus pneumoniae	−	−	−	+						
Vibrio comma	−	+								
Chromobacterium violaceum	−	+								
Eberthella typhi	−	−	+							
Eberthella paradysenteriae Hiss	−	−	+							
Eberthella paradysenteriae Flexner	−	−	+							
Salmonella enteritidis	−	−	−	+						
Salmonella morgani	−	−	−	+						
Salmonella paratyphi	−	−	−	+						
Salmonella suispestifer	−	−	−	+						
Salmonella schotmulleri	−	−	−	+						
Salmonella pullorum	−	−	−	+						
Salmonella gallinarum	−	−	−	+						
Escherichia coli	−	−	−	−	−	−	+			
Aerobacter aerogenes	−	−	−	−	−	−	+			
Phytomonas bowlesii	−	−	+							
Saccharomyces marianus	−	−	−	−	−	−	−	−	−	−
Saccharomyces intermedius	−	−	−	−	−	−	+			
Saccharomyces ellipsoideus	−	−	−	−	−	−	−	+		
Saccharomyces pastorianus	−	−	−	−	−	−	−	−	+	
Schizosaccharomyces mellacei	−	−	−	−	−	−	+			
Zygosaccharomyces priorianus	−	−	−	−	−	−	−	+		
Torula sphaerica	−	−	−	−	−	−	−	−	−	−
Aspergillus niger	−	−	−	−	−	−	−	−	−	−
Aspergillus ochraceus	−	−	−	−	−	−	−	−	−	−
Rhizopus nigricans	−	−	−	−	−	−	−	−	−	−
Alternaria sp	−	−	−	−	−	−	−	−	−	−
Fusarium sp	−	−	−	−	−	−	−	−	−	−
Penicillium sp	−	−	−	−	−	−	−	−	−	−

others are not influenced by the surface growth. The species which showed the most marked inhibition were the spore forming organisms from soils and various micrococci. *B. coli* and *Serratia marcescens* were not inhibited. Results obtained by this method are shown in the accompanying illustration. Plain extract agar was seeded with the various organisms and inoculated on the surface with *Pseudomonas fluorescens*. The cultures were seventy-two hours old when photographed (figs. 1–4).

It was deemed desirable to determine not only whether or not a given species is inhibited by the metabolic products but also the minimum amount of such products necessary to cause total inhibition of growth. Accordingly a series of agars was prepared each containing a different per cent of aged medium. The culture of *Ps. fluorescens*, grown on the surface of sloped Uschinsky agar for a period of ten days, was sterilized at 15 pounds pressure and added to the nutrient agar base. The effect on a variety of test species is shown in the accompanying table 1.

It is seen that the antagonistic action is not specific but that some species are more resistant than others. This is especially true of *Ps. fluorescens* and *Ps. pyocyanea*. The most sensitive species were found to be *B. anthracis*, *B. megatherium*, *Vibro comma*, *Chromobacterium violaceum* and *Rhodococcus*. Other species of *Bacillus*, *Eberthella*, *Sarcina*, *Neisseria* and *Phytomonas* are only slightly more resistant. The species of *Salmonella* are less sensitive while *Escherichia coli*, *Aerobacter aerogenes* and *Serratia marcescens* are relatively strongly resistant. All of the fungi grew normally while some of the yeasts were inhibited by amounts of 20 to 50 per cent.

Further evidence on the sensitiveness of bacteria and fungi was obtained by plating soil dilutions in the various grades of agar, prepared as above plus 0.5 per cent glucose. On plates prepared from dilutions of 1:1000 and 1:10,000, colonies of bacteria, except green fluorescent species, failed to develop on the grades above 20 per cent. The number and variety of mold colonies was practically the same as on the acid synthetic agar of Waksman's formula. Actinomycetes appeared to be more sensitive than fungi since very few colonies were observed. It

appears probable that all bacteria except the green fluorescent forms are inhibited while fungi do not appear to be affected. It is obvious that inhibition is not due to the reaction of the medium or to lack of food but to a thermostable toxic substance.

It seems probable that differences in resistance to the toxin depend, at least in part, on varying degrees of permeability. It is known that the cell wall of *B. cereus* is, in general, much more permeable than that of *B. coli*. Shaughnessy and Winslow (1927) have suggested that this phenomenon is probably related to the unequal periods of survival of these organisms in aqueous suspensions. The precise chemical nature of the bacterio-toxic substance produced by *Ps. fluorescens* is not known. It is water soluble and dialyzable through collodion and cell membranes and inhibits *B. cereus* and related forms in less amounts than are required for *B. coli*.

CONDITIONS WHICH INFLUENCE PRODUCTION OF THE TOXIN

It is well known that the development of true bacterial toxins depends on the composition of the culture medium and growth conditions. Previous investigations by Gunst (1923) and Lode (1903) have shown that the production of inhibitory and bactericidal substances by other species is influenced by the amount of available oxygen.

In order to test this effect, cultures of *Ps. fluorescens* were grown in Uschinsky's solution so arranged as to afford different exposures of air. The maximum exposure was obtained by placing 50 cc. of the medium in a 500 cc. Erlenmeyer flask. A similar flask was filled well up in the neck for comparison. The surface of the medium in each flask became covered with a dense zoogleal mass. The green fluorescent pigment developed more promptly in the shallow layer but at the end of the incubation period it had faded to a deep reddish green while there was little or no change of color in the deep flask. On agar containing 5 per cent of sterilized medium from the shallow layer, none of the test species grew while all of the same species developed on agar prepared by dissolving 0.5 peptone, 0.3 beef extract and 2.0 per cent of agar in the undiluted staled medium from the deep

layer. Proper aeration appears therefore to be a most important factor in the development of the toxic substance.

EFFECT OF VARIOUS AGENTS ON STABILITY

The bacterio-toxic substance produced by *Ps. fluorescens* is thermostable. Old agar cultures were sterilized at 100° and 120°C. for fifteen minutes and at 120°C. for thirty minutes. On nutrient agars containing 5.0 per cent of each, the sensitive species failed to develop.

The effect of desiccation was tested by saturating filter paper with Uschinsky's solution in which the organisms had grown for fourteen days. As soon as the paper became thoroughly dried the minimum amount necessary to cause complete inhibition was determined by adding various sized pieces to nutrient agar which was then sterilized and inoculated with a variety of species. A strip two by three inches added to 25 cc. of agar was sufficient to inhibit all of the spore formers and the sensitive cocci. The saturated paper was stored at temperatures of 58°, 37°, 30° and 10°C. and tested at intervals of ten days for a period of forty days. At the end of this period no deterioration had occurred.

The toxin is quite stable with age. Cultures grown on sloped Uschinsky agar were kept at room temperature for a period of thirty days and in the ice box for an additional sixty days. Evaporation was prevented by sealing with paraffin after the cultures were about three weeks old. On agars containing various amounts of the aged culture it was found that 3.5 per cent completely inhibited growth of all the spore formers and micrococci. The liquid medium loses its toxin more rapidly than agar. A culture kept forty-five days at room temperature had become completely non-toxic.

The toxin is not removed by filtration through Berkfeld or Seitz filters. Inhibition of the same species occurred when filtered or heated broth was added to nutrient agar in equal amounts.

BACTERICIDAL EFFECTS

Sterilized cultures grown in Uschinsky's solution failed to exert bactericidal effects against any of the species tested. In order

to test the effect of the unheated toxin, bits of agar removed from the inhibited zone of seeded plates were planted in nutrient broth. None of the spore forming species were killed while *Staph. albus Staph. aureus, Staph. citreus, B. typhosus B. paratyphosus A, B. paratyphosus B, Rhodococcus cinnibar* and *Sarcina lutea* failed to develop after a period of three to five days.

PRODUCTION OF TOXIN IN MANURED SOIL

The investigations of Conn (1917) and Conn and Bright (l. c.) have shown conclusively that *B. cereus* is not able to compete successfully with *Ps. fluorescens* in manured soils. To what extent this failure is due to the inhibiting metabolic products is open to question. It seems reasonable to suppose that under optimum conditions for the growth of *Ps. fluorescens* such inhibition might become pronounced. The very small amount of aged medium necessary to cause total inhibition of growth indicates that this is a probable explanation.

The hypothesis that soils frequently contain toxic substances which inhibit the growth of the higher plants and soil microorganisms appears to be quite generally accepted. It is known that such toxic effects may be removed by various methods of treatment such as oxidation, partial sterilization by heat, treatment with volatile antiseptics, the addition of certain substances and the adsorptive action of carbon black.

The precise mode of origin of soil toxins has not been conclusively demonstrated although there seems to be a more or less uniform opinion that such substances are not excreted as such by the roots of higher plants. It seems quite probable that soil microörganisms play an important part in their formation. Various workers have made comparative studies on the toxicity of soil extracts and bacteriotoxic substances produced by organisms when grown on artificial culture media. Frost (1904) was unable to obtain bactericidal effects against *B. typhosus* by any of the soil extracts tested but when pure cultures of various species isolated from the same soils were grown in broth, the effect was pronounced. He concluded that bactericidal sub-

stances do not exist preformed in soils, but are produced by various soil bacteria in artificial cultures.

Greig-Smith (1914) found that soils at times contain substances which are not favorable for the growth of *B. prodigiosus*. Extracts from the surface layer proved to be thermolabile while the sub-soil extracts were thermostable. His experiments showed that the soils with which he worked contained a variable bacteriotoxic content. Following rains which washed the substances down into the sub-soil the surface layers become non-toxic. The toxins were formed again as the soil dried out and persisted so long as suitable soil moisture was present. In very dry soil the toxin decayed. Experiments in the laboratory showed that a soil originally toxic became nontoxic when washed with water and upon incubation again became toxic. Such results would appear to point strongly, if not conclusively, to the origin of the bacterio-toxic substances through the activity of soil microorganisms.

In subsequent investigations Greig-Smith (1918) compared the effect of substances produced by microörganisms in cultures grown under various conditions with the soil extracts previously reported. Such inhibitions of *B. prodigiosus* as became manifest could be accounted for by changes in the hydrogen ion concentration of the medium and differed therefore from the toxic effects noted in soil extracts.

Hutchinson and Thaysen (1918) examined several samples of soil for the purpose of determining whether or not the beneficial effects of partial sterilization are due to the destruction of bacterio-toxins. Some of the soil extracts were found to be more suitable for the growth of *B. prodigiosus* than others. The unfavorable extracts were improved by the addition of peptone or antiseptics but not by heating. They found also that continued cultivation of *B. prodigiosus* in a solution produces an unfavorable medium which is not improved by heating. They regard this substance as different from the soil toxins reported by others.

In order to determine whether the toxin is produced in manured soils, cultures of *Ps. fluorescens* were prepared in soil to which 5.0 per cent of cotton seed meal or fresh horse manure was added.

The manured soil was placed, in 100 gram amounts, in Erlenmeyer flasks of 500 cc. capacity, sterilized by heating two hours at 15 pounds pressure, inoculated with *Ps. fluorescens* and incubated at room temperature for a period of fourteen days. Water extracts were prepared by adding 100 cc. of distilled water, shaking thoroughly at ten minute intervals for about two hours and then filtering through paper. Agar was prepared from the filtrates by the addition of 0.5 per cent peptone, 0.3 per cent beef extract and 2 per cent agar. The finished medium was sterilized at 15 pounds pressure and inoculated with various test species.

The manured soils failed to yield toxic substances in sufficient amount to inhibit the most sensitive of the test organisms. This seems to indicate that the bacterio-toxic substance is not produced under the conditions established or that it is adsorbed by substances in the soil and is not readily extracted by water.

It is known that soil toxins as well as the bacterio-toxins produced in cultures are adsorbed by charcoal and other substances. Gundel (1927) found that a thermolabile, non-filterable inhibiting substance produced by *B. coli* and antagonistic to *B. anthracis* is adsorbed by charcoal and several other compounds. Pratt (1924) found that the inhibitory effect of broth aged by *Fusarium* is removed by charcoal.

The power of charcoal and of field soil to adsorb the toxin from old cultures of *Ps. fluorescens* grown in Uschinsky solution has been tested in this investigation. The substances were placed in flasks and saturated with the solution. After standing twelve hours a quantity of distilled water equal to that of the original aged culture medium was added. The flasks were shaken thoroughly and the contents filtered through paper. The filtrates had lost most of the pigment which was originally present. This was especially pronounced in the case of the charcoal. Agars were prepared by dissolving the standard amounts of peptone, beef extract and agar at 15 pounds pressure. All test organisms, including the sensitive spore formers and cocci, developed normally thus showing that the toxic substance is not readily extracted from soils in which it is known to be present in abundance.

It is possible to extract some of the adsorbed toxin by means of alcohol. The charcoal was dried at 60°C. and extracted twelve hours. The filtrate obtained by filtration through paper was evaporated to dryness at 60°C. The dry residue was then redissolved in nutrient broth. On agar prepared from the broth the more sensitive species such as *B. cereus, B. mycoides, B. anthracis* and *Sarcina lutea* failed to grow. *B. coli* and *Serratia marcescens* were not inhibited.

With these data at hand, a second attempt was made to extract the toxin from sterilized manured soils which had been inoculated with *Ps. fluorescens*. The extracts were prepared in the manner described above. The alcoholic filtrate obtained from 100 grams of soil was evaporated to dryness at 60°C. and redissolved in 25 cc. of distilled water. On nutrient agar prepared from this water and standard amounts of nutrients, the more sensitive species failed to grow, while resistant forms such as *B. coli* and *Serratia marcescens* were not inhibited. Control flasks containing the same soil, sterilized but not inoculated, failed to yield toxin. Soil extracts prepared in the same manner from ten samples of field, garden, pasture and woodland soils were not toxic. None of these soils had been freshly manured and none contained large numbers of *Ps. fluorescens* cells.

Whether or not the toxin produced by *Ps. fluorescens* in soils is adsorbed to such a degree as to neutralize its inhibitory effect on bacteria in the soil might be questioned. Evidence on this point was obtained by sterilizing cultures of *Ps. fluorescens* which had grown in manured soil for a period of fourteen days and reinoculating with *B. cereus*. Control flasks of sterile soil were inoculated for comparison. Quantitative analyses were made at daily intervals to determine relative rates of multiplication. The soils in which *Ps. fluorescens* had grown proved to be as favorable for the growth of *B. cereus* as the uninoculated controls. Within 24 hours from the time of inoculation the number of cells had increased from about 30,000 to 1,250,000 per gram.

This result fails to support the theory that the toxic metabolic products exert the same effect in soils as they do when added to artificial culture media. It is, however, in complete harmony

with the experiments on adsorption. It appears improbable that other species of soil microörganisms are more potent in the production of bacterio-toxin than the strain of *Ps. fluorescens* employed in these experiments. The evidence fails to warrant the conclusion that the failure of *B. cereus* to compete successfully with *Ps. fluorescens* in manured soil is due to the toxin produced by the latter. The theory that free bacterio-toxins accumulate in soils in sufficient amounts to inhibit the growth of bacteria seems very doubtful. It appears to be much more probable that difference in nutritive requirements and relative growth rate rather than inhibition due to toxic metabolic products accounts for the difference in number of cells noted by others.

SUMMARY

1. *Pseudomonas fluorescens* produces a thermostable, filterable dialyzable bacterio-toxin which is both inhibitory and bactericidal.

2. The toxin is not specific in its action but is more active against certain species than others. Fungi are more resistant than bacteria. Spore-forming bacteria and micrococci are very sensitive, while colon bacteria are resistant. The substance is not isoinhibitory.

3. The amount of toxin produced depends on the composition of the culture medium and availability of oxygen. Maximum production occurs in cultures grown on the sloped surface of Uschinsky's agar.

4. The toxin persists longer in agar than in broth cultures and resists desiccation for long periods of time. It is soluble in alcohol, and is adsorbed by substances in soil and by charcoal.

5. The toxin is produced in sterilized manured soils inoculated with *Ps. fluorescens*. Alcoholic extracts from such soils are inhibitory for the more sensitive species only.

6. Soils which have supported a vigorous growth of *Ps. fluorescens* in flask cultures are suitable for the growth of *B. cereus* after sterilization.

7. Samples of soil from fields, gardens, pastures and meadows failed to yield toxins by the same methods of extraction employed for the pure cultures. It appears that the toxin studied here is

of a different nature from that of the thermolabile substances reported by others from soils.

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BACTERIAL CALORIMETRY

I. GENERAL CONSIDERATIONS. DESCRIPTION OF DIFFERENTIAL MICROCALORIMETER

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The production of heat by living bacteria is one of the transformations of energy brought about by these organisms. Although the rise in temperature of fermenting liquids, decomposing manure and other organic substances has been studied on account of its economic effects, and although, periodically, investigations of heat production in relation to the energetics of microörganisms have been made, it is not possible at this time to state definitely the significance of this liberation of heat or to correlate it with the various phases of the growth and activity of bacteria. In fact, the most recent and valuable compilation of this knowledge by Buchanan and Fulmer (1928) contains a paragraph on the utilization of energy in the development of heat which concludes with this sentence: "More complete studies on the utilization and partition of energy in the growth of microorganisms are needed."

The rise in temperature of organic substances undergoing bacterial decomposition has been called "thermogenesis." Its importance is connected chiefly with so-called "spontaneous heating," which is useful in some agricultural processes, but harmful in others, particularly those in which destructive combustions occur. Within the past year, James (1927–1928) has investigated this type of thermogenesis and has published a comprehensive review of the literature on the subject. These studies, however, have been concerned primarily with increase in tem-

perature and do not afford data on heat production of the kind which can be used in the investigation of energy exchange.

In considering the energy transformations of microörganisms the assumption is made that, when light is excluded, these organisms derive from the aerobic or anaerobic oxidation of foodstuffs all the energy required for their growth, structure formation, maintenance, repair, reproduction, motility, physical states and all the work associated with their vital processes. There is no evidence that they receive energy from other sources. Absorption of heat from the environment affects their chemical and physical reactions, but this heat is not a source of energy to the minute isothermal cell. Hill (1912) has clearly demonstrated the fallacy of regarding bacteria as heat engines, as was suggested by Simonson (1910).

On this assumption, attempts have been made to strike the energy balance of bacterial processes on a calorimetric basis. Rubner (1902, et seq.), as a part of his epochal investigations of the energy transformations of living organisms, measured the heat produced by bacteria. Rubner's bacterial calorimeter was a vacuum flask, carefully insulated and fitted with a delicate thermometer. In order to arrive at an energy balance he determined the heat liberated by the bacteria, the combustion caloric values of the uninoculated medium, the residue medium after growth and the total growth or "crop" of bacteria. In spite of the difficulties and uncertainties of corrections which must be used to compensate for effects produced by changes in the environmental temperature of this type of calorimeter, Rubner obtained important results, which will be discussed later. In a somewhat similar manner, omitting the direct measurement of heat produced, Tangl (1903) and Terroine and his associates (1922, et seq.) have attempted to discover how much energy microörganisms require for the formation of their structure. It does not seem to be probable that combustion values of foodstuffs, residue and bacteria can furnish the information required for the solution of these problems. Among various factors which are not taken into account by this method is the free energy of the substances utilized. The free energy of foodstuffs may differ

considerably from the energy value derived from measurements of the heat produced on combustion. Oppenheimer (1925) has shown that the combustion values of foodstuffs are "practically" equal to their free energies. On the other hand, Baron and Pólányi (1913) and Linhart (1920), and others, have so clearly pointed out that the free energy of these substances must be taken into consideration that it would be unwise to rest modern studies of bacterial calorimetry upon the older values of energy obtained by combustion methods.

The most recent and valuable studies of bacterial calorimetry are those of Meyerhof, A. V. Hill and Shearer. By combined measurements of oxygen consumed and carbon dioxide liberated, together with measurements of heat produced, Meyerhof (1912) has determined the caloric quotient of some phases of bacterial growth. This quotient is expressed as the ratio: heat produced in calories/milligram of oxygen consumed. Through study of this quotient, Meyerhof discovered evidence of the extreme complexity of the factors involved in the production of heat in a culture of bacteria, showing that not all of the heat found in the cultures can be attributed to the oxidation processes of the bacteria. The contribution made by Hill (1911–1912) to the advance of this work has been most direct in the form of the useful differential microcalorimeter devised by him. The great value of his general investigations of the energetics of cellular processes will appear in all phases of this work. Shearer (1921), using Hill's calorimeter, discovered that bacteria liberate much less heat when utilizing free amino acids than when they use more complex protein split products.

It is not intended to review all of this literature here. It will be more advantageous to take up the consideration of appropriate papers in connection with experimental measurements of the heat produced by bacteria at various stages of their growth and activity. In general the literature shows that the physiologists have for a long time perceived the advantage of using bacteria as subjects for the investigation of the energetics of cells. Rubner's belief that the metabolism of bacteria follows the same laws as that of the cells of higher organisms has been justified by the discovery from the study of bacterial processes of laws govern-

ing the metabolism of the cells of higher organisms. For example, recent advances in the knowledge of the mechanism of the contraction of muscle and of the respiration of cancer cells (Warburg, 1927) owe a part of their success to the results of the investigations of the energy exchanges of bacteria.

From the point of view of the bacteriologist, however, some of the investigations of these physiologists seem to suffer from lack of attention to the strictly bacteriological aspects of the subject. Precautions to keep cultures from contamination have not always been adequate, and in some instances have not even been attempted. It is apparent also that, through ignorance or neglect of the phases of growth of microörganisms, some of the most interesting periods of metabolism have not been studied.

It is evident that one of the first requisites for the investigation of these problems is an apparatus by which heat production by bacteria may be measured accurately.

DESCRIPTION OF DIFFERENTIAL MICROCALORIMETER

A calorimeter for use in the measurement of heat production by bacteria should be sensitive to small differences in temperature in the apparatus and as independent as possible of temperature changes in its external environment. It should be so constructed as to permit sterilization of all parts in contact with the culture, should allow stirring of the culture without causing unequal changes in temperature and should be provided with suitable openings, protected against temperature disturbances due to evaporation, for the introduction of the inoculum and for the withdrawal of small samples of fluid and gases. The differential microcalorimeter described by A. V. Hill (1911–1912) is an apparatus suitable for the measurements of small amounts of heat and automatically eliminates errors due to small changes in the environmental temperature. Since my use of this apparatus for the measurement of the heat liberated by reactions of antigens with antibodies (Bayne-Jones, 1925) I have made some changes in it which permit sterilization of the culture flasks and secure protection against contamination. These modifications, together with the methods used in calibrating the apparatus will be described in the following paragraphs.

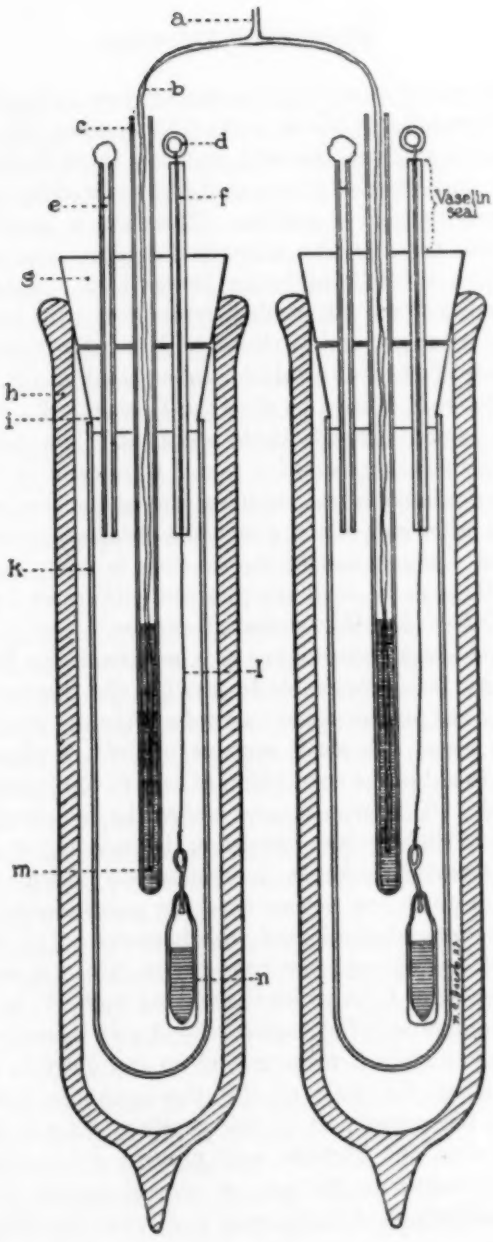

FIG. 1. SECTIONAL VIEW OF DIFFERENTIAL MICROCALORIMETER

The basic parts of the calorimeter are two cylindrical silvered vacuum Dewar flasks, 30 cm. long, 4.5 cm. wide, 380 cc. volume. Figure 1 is a sectional drawing showing these flasks and their contents. The Dewar flasks cannot be sterilized by heat without altering their thermal properties. Therefore, a vessel which can be filled with the requisite amount of medium and sterilized in the autoclave is fitted inside the Dewar flask. This vessel, *K*, is a rounded bottom, thin-walled pyrex glass tube measuring 20 by 4 cm. Its upper end is closed with a rubber stopper, *I*, or may be made of glass with suitable tubes fused into it. A second rubber stopper, *G*, is used to close the Dewar flask. Three glass tubes are passed through these stoppers. The tube marked *E* has an inside diameter of 3 mm. It is open at both ends, passes into the inner vessel, reaching almost the level of the fluid and has its outer end, 8 cm. above the outside stopper, plugged with cotton. Inoculation of the medium is made through this tube, and through it small samples are withdrawn by capillary pipette. Tube *C* is a thin-walled glass tube, 4 mm. in diameter, closed at the lower end to serve as a receptacle for the thermocouple. As it is impracticable to sterilize the thermocouples, *A* and *B*, they are placed inside this tube after the sterilization of the culture vessel. A small amount of fluid is placed in this closed tube to aid in the conduction of heat to the junctions of the thermocouple. This arrangement makes the temperature measuring system slightly less sensitive, introducing a short lag. These slight errors, however, having values which can be determined, are much less serious than the uncertainties produced by thermocouples contaminated with bacteria. The third tube, *F*, is a thick-walled capillary tube to provide a sleeve for the thread or fine wire, *L*, to which the stirring bulb, *N*, is attached. This bulb is composed of glass partly filled with mercury and is of such a size as to allow it to be moved up and down in the vessel without striking the wall of the thermocouple tube. After sterilization, the upper part of this capillary tube is filled with vaseline to shut out bacteria and prevent evaporation. The ring, *D*, is attached to the arm of an automobile wind-shield wiper, the oscillations of which raise and lower the bulb, *N*, stir-

ring the contents of the culture vessel. Bulbs of this type are more satisfactory than air bubbles or rotating blades for stirring the cultures. Air causes frothing of the culture medium, evaporation, temperature disturbances which cannot be controlled and irregular distribution of the bacteria. Rotating blades stir adequately. But it is very difficult to revolve the blades in each flask at the same rate and the mechanism for rotating the blades takes up more room than that used to raise and lower the bulbs.

Two Dewar flasks with sterilizable inner vessels compose the differential calorimeter. One vessel, into which is placed one junction of the thermocouple, contains the culture. The second vessel, serving as a control, contains the other junction of the thermocouple. The pair of vessels is so adjusted that temperature differences between them due to any other causes than the changes occurring in the culture are automatically almost completely balanced out. The temperature changes indicated by deflections of a galvanometer attached to the terminals of the thermocouples are, therefore, directly referable to changes in the culture. In order to protect the calorimeter against temperature disturbances from the outside, as an additional precaution, the flasks are submerged in a water bath, the temperature of which rarely varies more than 0.01°C., and often remains constant for many hours.

In order to balance out temperature changes due to fluctuations in the environmental temperature, the two flasks are given the same coefficient of thermal conductivity by placing an appropriate amount of fluid in each.

The conductivity constant for loss of temperature by a vacuum flask may be calculated from the following expression:

$$K \log e = \frac{1}{t} \log \left(\frac{A - T_0}{T - T_0} \right)$$

where K = conductivity coefficient for temperature loss.
t = time, usually in hours.
A = initial temperature.
T = temperature of fluid in flask at time t.
T_0 = temperature of environment (e.g., water bath).
$\log e$ = 0.434.

This coefficient K, varies with the amount of fluid in the flask. Hence K can be made to have any desired value within certain limits by increasing or decreasing the amount of fluid in the flask. After determining K for a standard volume of fluid in one flask, the other flask can be given an identical value of K by putting into it more or less than the standard amount of fluid. This amount of fluid can be determined from tables prepared when the calibrations are made.

Corrections for heat loss, which are used in the final computation of the total heat produced, are based upon the value of this coefficient of temperature loss, K. In order to calculate the total amount of heat produced during an experiment it is necessary to add to the observed heat production an amount equal to that which has been lost by conduction to the outside. The formula for this is given by Hill as follows:

Total heat liberated = (heat capacity of the flask and fluid) $(T - T') - (A - A')$ [K (area of the curve)]

where $T - T'$ = the observed difference in temperature between the two flasks;

$A - A'$ = the initial value of $T - T'$.

K = the coefficient of conductivity for loss of temperature. The value used is taken proportional to the interval of time for which the calculation is to be made.

These values are known from the observations.

The expression K (area of the curve) refers to the curve relating the difference in temperature between the two flasks to the time during which the observations are made. The area of the curve can be obtained with sufficient accuracy for any interval by multiplying the observed value $(T - T')$ in the middle of that interval by the duration of the interval in hours or fractions of hours. Therefore, K (area of the curve) = K [value of $(T - T')$] in the middle of the interval. This expression gives the temperature lost during the particular interval of time.

The loss of heat calculated thus for each interval, added to the loss calculated to the end of the previous interval and finally

added to the observed heat production, gives the total heat liberated.

The temperature changes are measured by means of thermocouples composed of junctions of copper and constantan wires. The constantan wire, B & S. No. 30 is 0.25 mm. in diameter, the copper wire, B. & S. No. 32 measures 0.202 mm. in diameter. These fine wires are silk-covered and are coated with shellac after completion of the junctions. The junctions are made by winding the copper around the constantan wire and fixing it with a small piece of solder. Elements composed of one to 10 junctions have been used. A single pair, or at most three pairs of junctions give sufficient electromotive force for the measurements to be made in this work. As an additional insulation and protection against wetting, the wires and junctions are enclosed in very thin capillary tubes, sealed at the bottom.

The copper wires leading from the thermocouple junctions are covered with rubber tubing and passed through glass tubes, well covered with rubber, to two pools of mercury in glass cups set into a block of wood. These pools of mercury are placed close together and protected as much as possible against unequal heating or cooling, to avoid thermoelectric effects at this point. Copper wires connect the mercury pools to the terminals of the galvanometer. Numerous tests have demonstrated that this system of wiring is free from parasitic currents arising from bimetallic junctions.

The galvanometer is a Type HS reflecting instrument made by the Leeds and Northrup Company. The total resistance of the galvanometer is 17.5 Ω. Its microvolt sensitivity is 4.5 mm. per microvolt at 1 meter and its external critical damping resistance is 50 Ω. The resistance of the galvanometer is approximately equal to the resistance of the thermocouples and circuit.

The readings indicative of temperature changes at the junctions of the thermocouple are taken directly from the scale divisions of the galvanometer deflection at 1 meter. No potentiometer is used. The calibration of the scale divisions in terms of temperature is made by numerous comparisons with Beckmann thermometers in the flasks with the thermocouple junctions.

Most of the thermocouples used give deflections in which 1 scale division = 0.003°C.

No experiment is begun until the pair of flasks composing the calorimeter are in temperature equilibrium with each other and with the water bath in which they are submerged. The Dewar flasks are rarely lifted out of the water bath, but are raised only high enough to allow removal of the inner vessels. After the inner vessels, containing sterilized culture medium, are brought from the autoclave they are placed in a pot of water until their temperature has cooled down to approximately that of the water bath. They are then placed in the Dewar flasks, submerged below the surface of the water bath, and at this time the thermocouples are let down into their appropriate tubes. The stirrers are connected and started. The whole system is left in this condition for ten to eighteen hours, which is the time required for the establishment of temperature equilibrium. At the end of this period, and after at least thirty minutes observation showing zero deflection of the galvanometer, the experiment is begun by the introduction of the inoculum into one of the flasks. With proper precautions, 1 ml. of a fluid culture may be introduced through tube *E* (fig. 1) by means of a capillary pipette without causing more than a slight change in temperature. Compensation for this is made by a slight shift of the zero of the galvanometer. Aseptic precautions at this time, and at subsequent removals of small amounts of the culture through tube *E* avoid contamination with undesirable bacteria.

CALIBRATION

Calibration of flasks

The determination of the value of the coefficient of temperature loss, *K*, is made by placing in a flask warm water, 1° to 2°C. higher than that of the surrounding temperature and allowing this to cool. Observations of the temperature at regular intervals of time provide data for the calculation of *K*. A number of such observations are made and an average value of *K* is taken from these results. The temperature may be measured with a

Beckmann thermometer or with the thermocouples. When the temperature in the flask is within 1°C. of the temperature of the water bath the cooling curve is in a region in which errors in measurement of the temperature have a larger effect than they

TABLE 1

Determination of K for flask No. 1, containing 100 cc. water

Values of A and T are expressed as the degree of temperature above that of the water bath.

t TIME	T_0 WATER BATH	A INITIAL TEMPERATURE	T TEMPERATURE AFTER INTERVAL	K
hours	°C.	°C.	°C.	
0	37.5	0.6405		
1	37.5	0.6405	0.528	0.1932
2	37.5	0.6405	0.453	0.1729
3	37.5	0.6405		
4	37.5	0.6405	0.333	0.1633
5	37.5	0.6405	0.285	0.1620
6	37.5	0.6405	0.242	0.1626

The value of K for each hour is calculated from the equation:

$$K \log e = \frac{1}{t} \log \left(\frac{A - T_0}{T - T_0} \right)$$

The average value of K per hour from these 5 determinations is 0.1708. Calculations of temperature corrected for loss by cooling are made as follows, using 0.1708 as the value of K per hour. The values for the temperature at the half-hour intervals are taken from the graph.

TIME	TEMPERATURE OBSERVED	$K \times$ VALUE OF MID. ORDINATE	TOTAL TEMPERATURE LOSS	CORRECTED TEMPERATURE
hours	°C.			°C.
0	0.6405	0	0	0.6405
1	0.528	0.099	0.099	0.627
2	0.453	0.083	0.182	0.635
3	0.395	0.071	0.253	0.648
4	0.333	0.061	0.314	0.647
5	0.285	0.053	0.367	0.652
6	0.242	0.045	0.412	0.654

do in cases in which the temperature difference is greater. Nevertheless, the equation for the calculation of the coefficient of temperature loss holds. An illustration of the method of determining K and of the calculations for corrections for loss of temperature is

given in table 1 and the accompanying graph, figure 2. The values of the corrected temperature lie on an approximately straight line parallel to the time axis at the level of the value of the initial temperature.

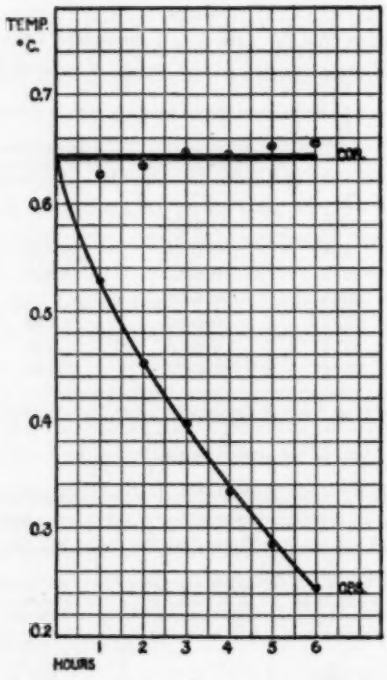

FIG. 2. GRAPH OF COOLING CURVE OF CYLINDER AND INNER VESSEL

Determination of sensitivity and accuracy

By liberating a small known amount of heat from a resistance coil in the fluid in one of the flasks it is possible to determine the sensitivity and lag of the system and to evaluate the errors in the measurements. A small coil of nichrome wire is placed in the fluid in one of the pair of differential flasks and a measured electric current is passed through it for a definite period of time.

A short lag occurs, but measurable changes in temperature are

detectable within a minute after the start of the flow of current. This interval is of no significance in the kind of work for which the apparatus is intended.

The heat produced by the coil is calculated from the formula:

$$H = \frac{RI^2}{4.18} \text{ gram cal. per sec.}$$

where H = heat produced.
R = resistance in ohms.
I = current in amperes.
4.18 = factor for converting joules into gram calories.

In one experiment the resistance of the coil was 11 Ω and the current 0.025 amperes. This produced 5.91 gram calories per hour. The corrected temperature at the end of five hours was 0.249°C. The heat capacity of the flask and fluid was equivalent to that of 125 cc. of water. Hence the observed heat production was 6.02 gram calories per hour. The error in this case was +1.88 per cent. In another experiment, lasting six hours, the observed heat production with the same coil and a current of 0.05 amperes was 22.89 gram calories per hour. The calculated heat production amounted to 23.68 gram calories. The error in this case was 3.33 per cent. These experiments demonstrate that the calorimeter and measuring devices are sufficiently sensitive and accurate to measure the production of a small amount of heat, of the order of magnitude of that produced by cultures of microörganisms, with an error of approximately ±3.0 per cent.

Experiments on the measurement of the heat produced by mixtures of definite volumes of absolute ethyl alcohol and water in the calorimeter gave results which differed only within this range of error from the heat production expected by calculation.

The heat capacity of the flask and its contents can be approximated from computations based upon the amount and kind of fluid in the flask, the specific heat of this, and the glass and other materials composing the vessels and apparatus in them. This estimate can be verified and corrected by calibrations in which a known amount of heat is liberated in the flasks. In general, the

heat capacity of the flasks and fluids used has been in the neighborhood of 125, e.g. equivalent to the heat capacity of 125 cc. of water.

As the methods of observation and computation which have been used in the experiments upon the heat produced by bacteria are applied in the case in which a known amount of heat is

TABLE 2

Calibration experiment

Dewar cylinder and vessel No. 1.
100 cc. water in vessel. Heat capacity of flask and contents = 125.
Coefficient of loss of temperature, K, = 0.1780 per hour.
Coil, resistance = 11 Ω.
Current 0.050 amperes. Gram. cal. per hour (calc.) = 23.68.
Thermocouple No. 3 gives 1 scale div. = 0.003°C.
Water bath temperature 37.5° ± 0.01°C.

HOURS	GALV. S.D.	°C. OBSERVED	$K \times$ VAL. MID. ORD.	TOTAL TEMPERATURE LOSS	°C. CORRECTED	HEAT GRAM CAL. OBS.	HEAT GRAM CAL. CALC.	DIF. GRAM CAL.	ERROR PER CENT
0	0	0	0	0	0	0	0	0	0
0.5	32	0.096							
1	58.8	0.1764	0.017	0.017	0.1934	24.22	23.68	0.54	+2.28
1.5	82	0.246							
2	105	0.315	0.0438	0.0608	0.3758	46.97	47.36	0.39	−0.82
2.5	127	0.381							
3	144.5	0.4335	0.0678	0.1286	0.5621	70.26	71.04	0.78	−1.09
3.5	163	0.489							
4	180	0.540	0.087	0.2156	0.7556	94.44	94.72	0.28	−0.29
4.5	194	0.582							
5	209	0.627	0.1035	0.3191	0.9461	117.26	118.40	1.14	−0.96
5.5	225	0.675							
6	235	0.705	0.120	0.4391	1.441	143.01	142.08	0.93	+0.65

liberated in the calorimeter, one of the calibration experiments is given in detail in table 2.

The curve of temperature changes which occurred in this experiment is shown in the accompanying graph, figure 3, in which temperature is plotted against time. The temperature, corrected for heat loss, falls on a straight line, ascending regularly, which is to be expected as the result of the continuous liberation of a constant amount of heat in the calorimeter. It is additional

evidence of the validity of Hill's formula for the calculation of the coefficient of temperature loss and shows the correctness of the value calculated for this case.

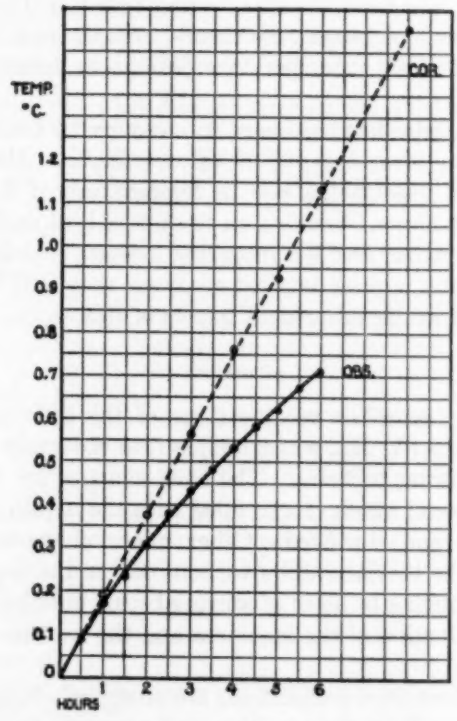

FIG. 3. GRAPH OF OBSERVED TEMPERATURE CHANGES PRODUCED BY HEATING COIL IN ONE VESSEL OF CALORIMETER

The corrected curve is a straight line, showing liberation of a constant amount of heat.

As an additional check on the ability of the calorimeter to eliminate automatically or balance out temperature changes due to warming or cooling the environment of both flasks, a pair of flasks, adjusted to have the same value of the coefficient of temperature loss, K, have been placed close together and left in posi-

tion for forty-eight and ninety-six hours. During this time, the temperature of the room or water bath rose and fell during intervals of two to ten hours through a range of about 2°C. The temperature difference within the flasks during this period, as measured by the thermocouple, varied between 0 and 0.012°C. These changes took place very slowly. Small rapid fluctuations produce no detectable temperature differences between a pair of balanced flasks.

This calorimeter has been used to measure the heat production of bacteria in several types of culture media. Most of these cultures have produced a rise in temperature of 0.2 to 0.5°C. in eight to ten hours, liberating an amount of heat easily measured by the calorimeter, and the recording system. As the curves of heat production require detailed analyses, they will be described and discussed in the subsequent papers of this series.

SUMMARY

This paper describes modifications of the differential microcalorimeter of A. V. Hill which adapts it to the study of heat produced by growing bacteria. The chief changes are the addition of an inner vessel which can be filled with the required amount of culture fluid and sterilized. Other minor changes permit the insertion of the thermocouples without contaminating the media, mechanical stirring in place of stirring by air bubbles, and a tube for the introduction of the inoculum and the withdrawal of small samples.

Data obtained from calibrations are presented showing that the apparatus is sufficiently sensitive and accurate to measure the production of approximately 6 gram calories per hour with an error of ±1.88 per cent and the liberation of 23.68 gram calories per hour with an error of ±3.33 per cent in a vessel and fluid having a heat capacity equivalent to 125 cc. of water. Heat production by cultures of bacteria is within these limits in most of the media employed and it is estimated that the error in the measurements within this range is approximately ±3 per cent.

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BACTERIAL CALORIMETRY

II. RELATIONSHIP OF HEAT PRODUCTION TO PHASES OF GROWTH OF BACTERIA

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Realizing the importance of establishing the time-relationships of a biological process, we have begun our investigations of bacterial calorimetry with experiments to determine the course of the production of heat during the growth of a culture of bacteria. At the points where the temperature was recorded we have counted the bacteria in the culture. From the results of these studies, which will be reported in this paper, we have been able to plot the curve of heat production with respect to time, discovering points of inflection of this curve, and have correlated the curves of heat production and of growth of pure cultures of several varieties of bacteria in different kinds of media. Quantitative data were secured as to the number of bacteria present at each unit of time and the gram calories liberated by them. Measurements of the sizes of the bacteria, determinations of their surface area and chemical studies of their metabolism were not made at this time as it seemed wiser to postpone these for future investigations which will be facilitated by being based upon a knowledge of the relationship of the production of heat to the phases of the growth of microörganisms.

Little definite information on this subject could be gathered from the literature, as the physiologists who have done most of the work in this field have overlooked a number of the significant phases of bacterial growth. Curves of heat production with respect to time appear in many papers. But in most cases the

long intervals of time between the observations passed over periods when the culture was undergoing great changes and in no papers have we found definite statements of the age and amount of the culture used as the inoculum or counts of the bacteria present at the moments when the temperature was noted. In view of the fact that genuine interest in the growth curves of microörganisms is comparatively recent, we would not expect to find attention paid to these points in the older literature of calorimetry. Among such papers, in which the course of heat production of cultures was noted without a simultaneous accumulation of enough bacteriological data to permit their analysis, may be placed those of Rubner (1911) and A. V. Hill (1911–1912). The graphs of heat production in souring milk, presented in Hill's paper, show that after a latent period of nine to ten hours, heat was produced at a rapid rate during the next four hours and thereafter at a reduced rate. In connection with Shearer's observations and our own, this change of the slope of the curve of heat production between the third and fourth hours after the beginning of active growth of a culture appears to indicate a definite metabolic change at that time. Shearer (1921), in a very interesting account of the way in which *B. coli* builds up amino acids into its protoplasm with relatively little waste of energy in the form of heat, presented a number of curves of heat production with reference to time. These curves show a short period of lag, with subsequent changes in the rate of liberation of heat at the third, sixth and twentieth hours. As Shearer did not attempt to determine the number of bacteria present at these points, it is impossible to refer his curves to actual growth curves. Our results have been quite similar, in general, to those obtained by Shearer, lending additional support to the assumption that these points of change in the slope of the curve are in some way definitely related to changes in the metabolism of the bacteria.

APPARATUS

The calorimeter used in these experiments was a modified form of the differential microcalorimeter devised by A. V. Hill (1911–1912). The modifications, thermocouples, galvanometer and

method of making measurements have been described in the first paper of this series (Bayne-Jones, 1929). The changes made in Hill's apparatus permit sterilization of the culture vessels and aid in avoiding contamination during the process of inoculation and withdrawal of samples. The error in the measurements was estimated to be ±3 per cent.

ENUMERATION OF BACTERIA

It is unnecessary to review all the recent literature on the subject of methods of counting bacteria. Our experience with plate-counts was unfavorable to the use of that method in connection with this work. For our observations, which extend over periods of eight to ten hours, we found that direct counting of bacteria in a Helber chamber 0.01 mm. deep, using a diluent containing basic fuchsin, together with an opacimetric determination upon a duplicate sample according to the method of Gates (1920) gave us reliable data from which growth curves could be constructed. It is admitted that direct counts and opacity determinations do not distinguish between living and dead bacteria, unless a special staining technic is used, such as that devised by Henrici (1923). On the other hand, it is well known that more than 90 per cent of bacteria in cultures less than twelve hours old are living and it may be presumed that nearly all the organisms in such a culture have some degree of vitality. In fact, Jensen (1928) regards the growth curves constructed from data obtained with his special method of direct counting as more representative of natural conditions than those based on the results of plate counts. Organisms in old cultures, occurring in clumps, and flakily growing organisms such as *B. subtilis* could not be counted by any method.

The small samples of the culture required for these counts were withdrawn by a sterile capillary pipette through a small tube passing through the rubber stoppers of the calorimeter vessel. Numerous tests demonstrated that no appreciable disturbance of the temperature of the culture was produced by this procedure.

GENERAL CONSIDERATIONS

The inoculum was introduced through this same tube at the beginning of the experiment. This usually consisted of 1 cc. of a twelve- to eighteen-hour broth culture of an organism. The culture was transplanted daily to reduce the period of lag following inoculation of the medium in the calorimeter. In these experiments, the period of lag was always less than one hour, and, in fact, evidences of heat production were often apparent fifteen minutes after the inoculation. Care was taken at all stages to ascertain by smears and plates that the material used for inoculation and the growth in the flask of the calorimeter were pure cultures of the organism in use. Whenever contamination occurred, the experiment was discarded.

As has been explained in the first paper, the medium in the calorimeter flask was always sterilized in the autoclave at least ten hours before the commencement of an experiment, and the medium and the calorimeter in the water bath were allowed to reach an equilibrium of temperature. While we attempted to have the amount of broth culture used for the inoculum at the same temperature as the medium in the calorimeter, we often failed to do this. On this account, differences of 0.003 to 0.01°C. were produced by the introduction of the inoculum. These small differences at the start of the experiment were usually discounted by a shift of the zero of the galvanometer attached to the thermocouples, and in any case had little or no influence upon the subsequent course of heat production by the organism.

With the exception of a few experiments made in 1926, the cultures were not aerated, but received oxygen through the small plugged tube passing through the rubber stoppers of the calorimeter. The condition within the culture flash was presumably slightly less aerobic than that in ordinary culture tubes plugged with cotton. At this time we did not attempt to study the gaseous exchanges of the organisms or the effect of oxygen tension upon heat production.

The temperature of the environment, which may be called the incubation temperature, was 37.5 ± 0.01°C. During most of the

experiments, the fluctuations of the temperature of the water bath were less than 0.01°C., usually within 0.005°C. as measured by a Beckmann thermometer.

CALCULATIONS

In the first paper of this series, the calculations used to determine the coefficient (k) of loss of temperature of the calorimeter flask have been set forth and experiments with heating coils have been recorded in detail to show how this factor was used in correcting the temperature. For most of the vessels, this coefficient was approximately 0.1780 per hour. In most cases, 100 cc. of culture medium were placed in the flask, which with its liquid and glass contents had a total heat capacity equivalent to 125 cc. of water. The corrected temperature multiplied by the heat capacity of the flask and contents gave the total gram calories of heat produced.

The heat produced by each bacterium was obtained directly by dividing the total gram calories by the number of bacteria present in the 100 cc. of culture medium. This value, of course, was actually too small to be measured, but some interesting relationships were discovered through the graphic use of it.

The same fact could be reached by a calculation. We are indebted to Dr. R. E. Buchanan for the formula for this calculation, having found it first in a note on the "Determination of the fermentation capacity of a single bacterial cell," published by him in Abstracts of Bacteriology in 1918. As a number of errors occur in that report, it seems advisable to present here the verified development of the correct formula which Dr. Buchanan sent us. The assumptions upon which the calculations are based are that the organisms are multiplying in geometric progression at a definite rate, and that each organism is excreting the substance, (or producing heat), at a definite uniform rate. The amount of the substance (or heat) produced must be measured at the end of each unit of time, and the number of bacteria counted at the beginning and at the end of each unit of time.

Let B = number of bacteria at beginning.

b = number of bacteria after time t.

n = number of generations in time t.
g = average generation time.
m = amount of substance (or amount of heat) produced per cell per unit of time.
S = total amount of substance (or heat) produced in time t.

Then

$$n = \frac{t}{g}$$

$$b = B\,2^n = B\,2^{\frac{t}{g}}$$

The amount of substance (or heat) produced during any instant dt would be $m \cdot b \cdot dt$. And the total amount (S) produced in time t would be

$$S = \int_0^t m \cdot b \cdot dt$$

Substituting

$$S = mB \int_0^t 2^{\frac{t}{g}}\, dt$$

upon integration

$$S = \frac{g\,m\,B\left(2^{\frac{t}{g}} - 1\right)}{ln\ 2}$$

Substituting:

$$S = \frac{Mt\,(b - B)}{ln\,\frac{b}{B}}$$

$$M = \frac{S\ ln\ 2}{g\,(b - B)} = \frac{S\ ln\,\frac{b}{B}}{t\,(b - B)}$$

Whence

$$M = \frac{S\ 2.303 \log \frac{b}{B}}{t\ (b - B)}$$

From the last expression, we have calculated the amount of heat produced by a single bacterium during each hour of the growth of the culture.

In accordance with the assumptions upon which the formula rests, the values obtained during the logarithmic period were most significant.

During the past two years, we have made a considerable number of experiments. The results of these have been in general so similar that several of them may be selected with propriety to illustrate the general principles with which this report is concerned. As conditions of media, size of inoculum, age of culture and other factors vary more or less in each experiment, a false conception of the relationship of the heat production to growth is given by an average of all results. Indeed, each individual experiment has a distinct validity. Nevertheless, we have averaged the results of six experiments with *B. coli*, which were almost identical, and will present this composite curve for comparison with the data of individual cases. Four cases will be presented as follows: heat production by (1) *B. coli* in a 2 per cent peptone broth, average of 6 experiments, (2) *B. coli* in 2 per cent Difco peptone water, (3) *B. coli* in 2 per cent Difco peptone water containing 1 per cent glucose and (4) *Staphylococcus aureus* in 2 per cent Difco peptone water.

All temperatures noted in the tables are expressed in terms of degrees Centigrade above that of the water bath. (See tables 1 to 4.)

TABLE 1

Heat production by B. coli in broth (average, plate 1)

Three hundred cubic centimeters of a broth containing 2 per cent Difco peptone— pH = 7.6.

Heat capacity of flask and fluid = 330.

Coefficient of temperature loss (K) = 0.1280 per hour.

Stirring by air saturated with water vapor.

Temperature of incubator = 37.5 ±0.1°C.

Inoculum = 1 cc. of eighteen-hour culture of *B. coli* (strain *S*) in this broth.

This table is composed of the average results of six experiments.

These results are shown graphically in figure 1, *A*, *B*, *C* and *D*.

TIME	TEMPERATURE CORRECTED	NUMBER OF BACTERIA PER CUBIC CENTIMETER × 10^9	LOG NUMBER BACTERIA PER CUBIC CENTIMETER	TOTAL HEAT GRAM CALORIES OBSERVED	TOTAL NUMBER BACTERIA × 10^9	GRAM CALORIES PER BACTERIA OBSERVED × 10^{-9}	GRAM CALORIES PER BACTERIA CALCULATED × 10^{-9}
hours	°C.						
0	0	1.2	6.07918	0	360.0	0	0
1	0.0045	1.4	6.14613	1.485	420.0	3.54	4.608
2	0.0536	7.2	6.85733	17.68	2160.0	8.182	8.77
3	0.1524	32.0	7.50515	50.29	9600.0	5.24	5.95
4	0.2323	112.0	8.04922	76.65	33600.0	2.28	2.65
5	0.2810	320.0	8.50515	92.73	96000.0	0.966	1.08
6	0.3291	430.0	8.63347	108.60	129000.0	0.842	0.829
7	0.3683			121.53			
8	0.4281			141.27			
9	0.4408			145.46			
10	0.4749			156.71			

TABLE 2

Heat production by B. coli in 2 per cent Difco peptone broth (plate 2)

One hundred cubic centimeters of peptone water containing 2 per cent Difco peptone and 0.5 per cent NaCl—pH = 7.6.

Heat capacity of flask and fluid = 125.

$K = 0.1780$ per hour.

Stirring by moving bulbs. Culture not aerated.

Inoculum = 1 cc. of eighteen-hour culture of *B. coli* (strain *S*) in the same medium.

Temperature of water bath = 37.5 ±0.01°C.

The results of this experiment are shown in the table and in the curves in figure 2.

TIME	TEMPERATURE		NUMBER BACTERIA PER CUBIC CENTIMETER $\times 10^6$	LOG NUMBER BACTERIA PER CUBIC CENTIMETER	TOTAL HEAT GRAM CALORIES OBSERVED	GRAM CALORIES PER BACTERIA OBSERVED $\times 10^{-9}$	GRAM CALORIES PER BACTERIA CALCULATED $\times 10^{-9}$
	Observed	Corrected.					
hours	°C.	°C.					
0	0	0	8.5	6.9294	0	0	0
1	0.009	0.0095	20.5	7.3117	1.19	0.581	0.491
2	0.0645	0.0716	45.4	7.6570	8.95	1.98	2.02
3	0.1065	0.1309	126.0	8.1003	16.3	1.295	1.25
4	0.123	0.1676	245.0	8.3891	20.9	0.855	0.742
5	0.1395	0.2075	330.0	8.5185	25.9	0.788	0.589
6	0.1545	0.2486	400.0	8.6020	31.0	0.776	0.51
7	0.1665	0.2891	480.0	8.6812	36.1	0.753	0.442
8	0.171	0.324	500.0	8.6989	40.5	0.736	0.235
9	0.1785	0.3627			45.2		
10	0.183	0.399			49.9		

TABLE 3

Heat production by B. coli in 1 per cent glucose (plate 3)

One hundred cubic centimeters of 2 per cent Difco peptone water, with 0.5 per cent of NaCl and 1 per cent glucose — pH = 7.6.

Heat capacity of flask and fluid = 125.

K = 0.1780 per hour.

Stirring by moving glass bulbs. Culture not aerated.

Inoculum = 1 cc. of eighteen-hour culture of *B. coli* (strain *S*) in this medium.

Temperature of water bath = 37.5 ±0.01°C.

The results of this experiment are shown in the table and in the curves in figure 3.

TIME	TEMPERATURE		NUMBER BACTERIA PER CUBIC CENTIMETER $\times 10^9$	LOG NUMBER BACTERIA PER CUBIC CENTIMETER	TOTAL HEAT GRAM CALORIES OBSERVED	GRAM CALORIES PER BACTERIA OBSERVED $\times 10^{-9}$	GRAM CALORIES PER BACTERIA CALCULATED $\times 10^{-9}$
	Observed	Corrected.					
hours	°C.	°C.					
0	0	0	8.64	6.9375	0	0	0
1	0.0375	0.0391	13.28	7.1232	4.88	3.68	4.5
2	0.114	0.1247	42.0	7.6232	15.57	3.71	3.7
3	0.198	0.2402	316.0	8.4996	30.0	0.95	1.17
4	0.246	0.3288	360.0	8.5563	41.05	1.14	1.087
5	0.279	0.4085			51.02		
6	0.294	0.4746			59.15		
7	0.303	0.5371			67.13		

TABLE 4

Heat production by Staph. aureus in 2 per cent Difco peptone water (plate 4)

One hundred cubic centimeters of 2 per cent Difco peptone water containing 0.5 per cent NaCl—pH = 7.6.

Heat capacity of flask and fluid = 125.

$K = 0.1780$ per hour.

Stirring by moving glass bulbs. Culture not aerated.

Inoculum = 1 cc. of eighteen hour culture of *Staph. aureus* in the same medium.

Temperature of water bath = 37.5 ±0.01°C.

The results of this experiment are shown in this table and in the curves in figure 4.

TIME	TEMPERATURE		NUMBER BACTERIA PER CUBIC CENTIMETER $\times 10^8$	LOG NUMBER BACTERIA PER CUBIC CENTIMETER	TOTAL HEAT GRAM CALORIES OBSERVED	GRAM CALORIES PER BACTERIA OBSERVED $\times 10^{-9}$	GRAM CALORIES PER BACTERIA CALCULATED $\times 10^{-9}$
	Observed	Corrected.					
hours	°C.	°C.					
0	0	0	6.62	6.8208	0	0	0
1	0.015	0.0157	24.0	7.3802	1.96	0.817	1.452
2	0.0564	0.0626	77.0	7.8864	7.82	1.01	1.365
3	0.0939	0.1112	159.0	8.2014	13.9	0.874	0.965
4	0.120	0.1572	293.0	8.4668	19.7	0.673	0.653
5	0.144	0.205	362.0	8.5587	25.6	0.77	0.583
6	0.159	0.246	378.0	8.5774	30.7	0.81	0.559
7	0.177	0.295	426.5	8.6299	36.8	0.863	0.522
8	0.1845	0.336	547.0	8.7379	41.9	0.766	
9	0.195	0.380	656.0	8.8169	47.5	0.724	

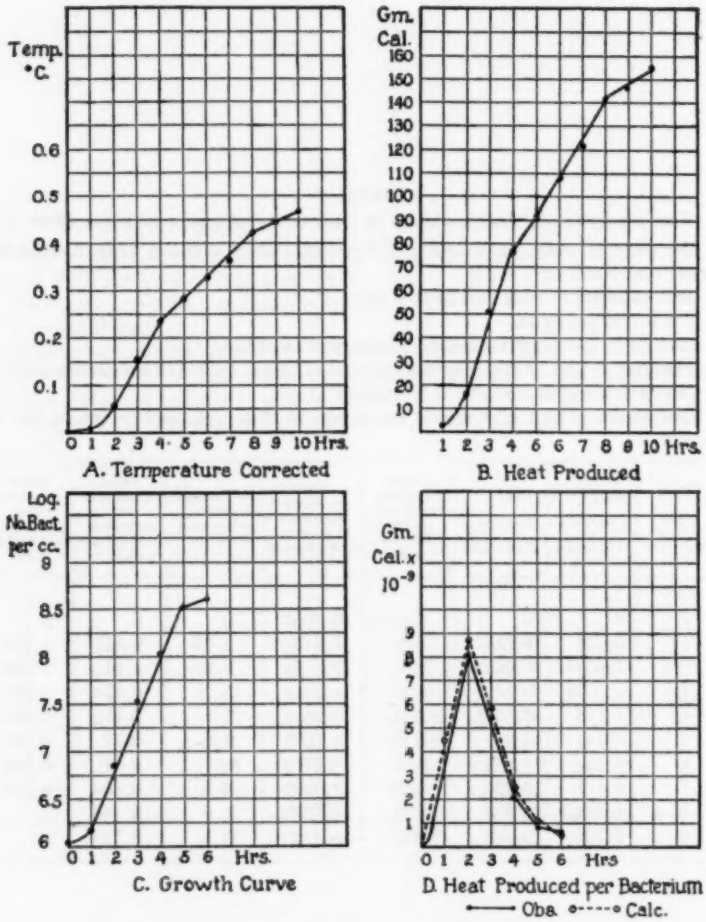

FIG. 1. GRAPHIC REPRESENTATION OF DATA IN TABLE 1

B. coli in peptone broth, aerated. Average of six experiments. Each figure (figs. 1–4) contains four panels showing curves of temperature, total heat production, number of bacteria per cubic centimeter and the heat produced per bacterium at intervals of one hour during the duration of the experiment.

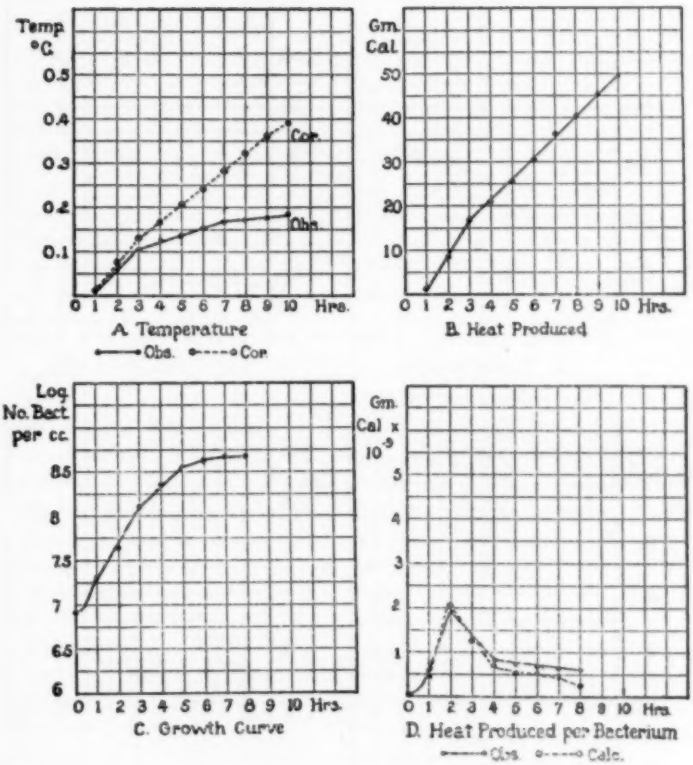

FIG. 2. GRAPHS OF DATA IN TABLE 2

B. coli in 2 per cent peptone water

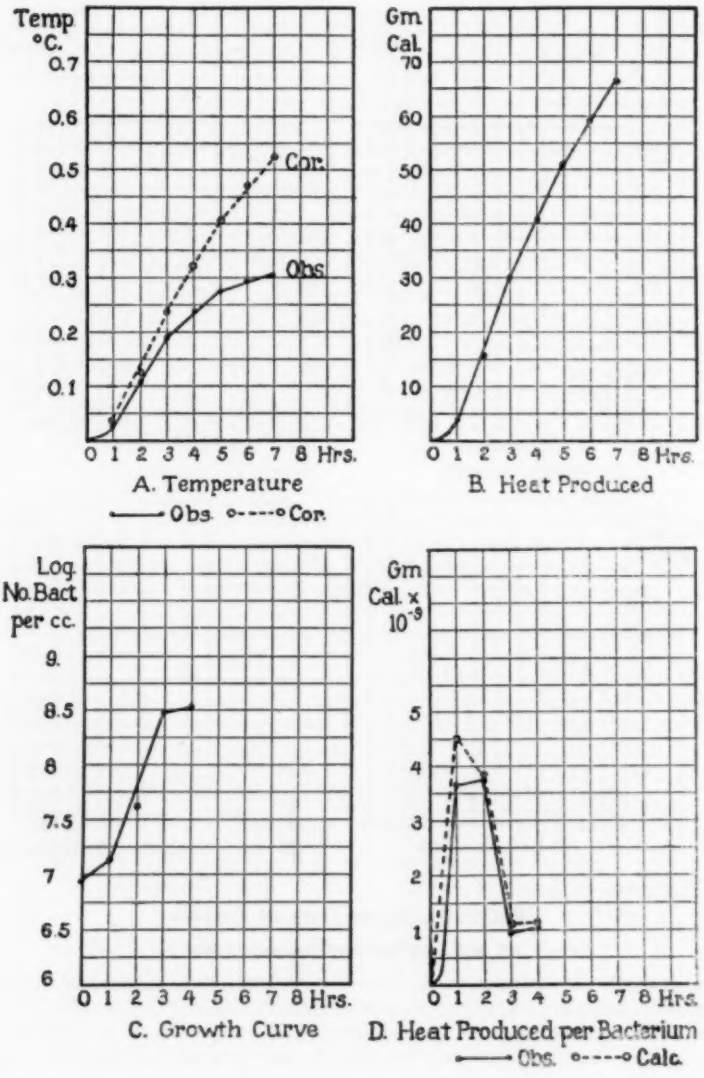

FIG. 3. GRAPHS OF DATA IN TABLE 3

B. coli in 2 per cent peptone water with addition of 1 per cent glucose

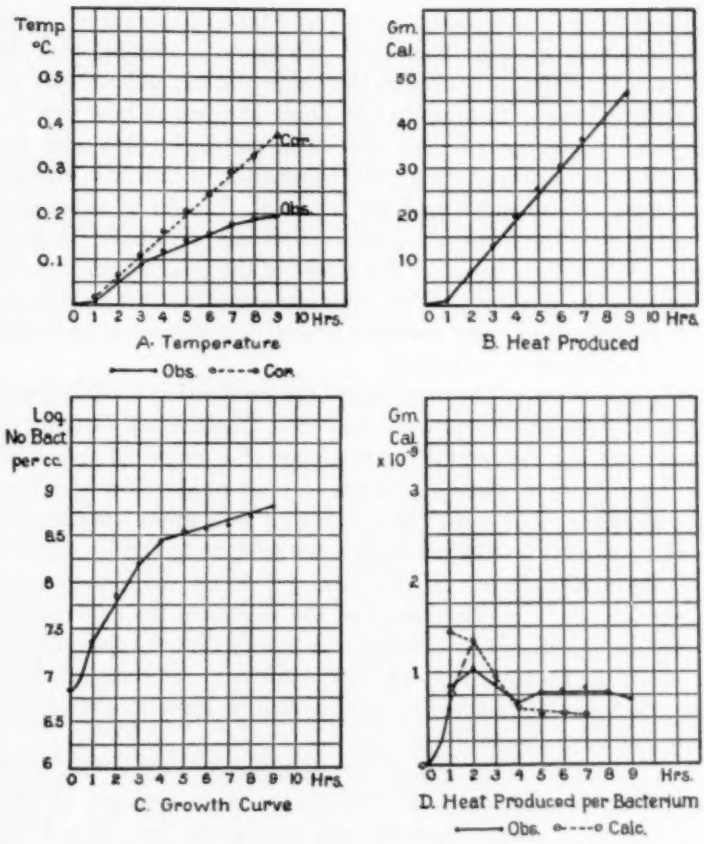

FIG. 4. GRAPHS OF DATA IN TABLE 4.

Staphylococcus aureus in 2 per cent peptone water

DISCUSSION

Without a full knowledge of all the factors it is obviously fruitless to seek an explanation of the shape of the curve of heat production by growing bacteria. We know in general that this heat has its ultimate source in the oxidation of foodstuffs by the microörganisms. But we do not know at present how much of this heat is directly referable to cellular metabolic processes, and how much is produced by physical processes of motility, changes in surface relationships and the heats of dilution of substances together with the heats of neutralization of acid or basic by-products. As bacteria grow in the midst of the food which they are using and the products which they are excreting, they evidently must give rise to a very complex interacting system of endothermic and exothermic reactions.

It is noteworthy, however, that heat production by cultures of bacteria is an orderly process and that Buchanan's formula for calculating the fermentation capacity of a single bacterial cell fits the curves in those instances of heat production which we have studied. The use of this equation is of great assistance in checking results and in reaching a decision as to the significance of unexpected values. At this time, we do not wish to give the impression that the coincidence of the results of the experiments with those calculated by this formula signify that the assumptions on which the formula were based are correct. It suggests, however, that during the logarithmic period of growth, each cell is liberating heat at a uniform rate.

One conspicuous feature of the observed as well as the calculated heat production is the relatively large amount of heat produced by a young bacterial cell, during the first three hours of growth, as compared with older cells, after the fourth hour of growth. Young cells produced several times as much heat per cell as older ones. During this period the cells are multiplying rapidly, and are large, as Henrici (1923, 1925) has shown. In fact, the shape of the curve during this period is almost identical with that of the index number of the area-length ratio plotted by Henrici, and is of the same shape as, but opposite in sign to, the curve of the index number of the average length. These

agreements still further relate morphological, functional and metabolic states.

In the tables of measurements and in curves drawn to a larger scale than those illustrating this paper, changes in the rate of heat production are discernible corresponding in general to the periods of lag, logarithmic phase, phase of negative acceleration, maximum stationary phase, and phase of decrease. Similar changes in curves of acid production have been noted. Changes in the rate of heat production have occurred invariably at about the end of the first hour, between the third and fourth hour and at about the fifth hour in these rapidly growing cultures. Similar sudden changes in the slope of the time: potential curves of several organisms were found by Clark (1926) and his associates during their studies on oxidation and reduction. These are undoubtedly critical points of fundamental significance in the metabolism of microörganisms, though we are unable to do more than locate them at present.

The studies of cytomorphosis of bacteria by Henrici (1925) and Clark (1928) show clearly that older bacteria are smaller than younger ones, change very little in shape in a short period, and, of course, almost cease to reproduce. This is a fairly long, if indefinite period. These studies of heat production indicate that a period of greatly reduced metabolic activity on the part of each bacterium begins at about the fourth or fifth hour and is maintained at a low constant rate for ten hours, and probably for a longer time. The curves in the illustrations of this paper clearly show this prolonged constant heat production per cell. It is suggested that this phase of heat production may be regarded as a basic metabolic rate of bacteria, if subsequent studies give results which justify the use of such a term as basic metabolism in connection with bacteria.

SUMMARY AND CONCLUSIONS

By means of simultaneous observations of the heat production by bacteria and enumeration of the organisms in the culture it is possible to correlate growth curves with curves of the liberation of heat. A combination of such observations, with those of the morphological changes and life-phases of bacteria, yield some

information as to the metabolic activities of a culture at various periods. By the use of Buchanan's formula, the rate of heat production by a single bacterial cell can be calculated, and the observed values agree closely with those derived by this calculation.

Changes in the rate of heat production are related to changes in the growth rate of bacteria.

Young bacterial cells produce more heat per cell than older ones.

After about the fifth hour of growth, the production of heat reaches a low level and remains constant, suggesting the existence of a basal metabolic rate during that period.

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NOMENCLATURE OF THE ROOT-NODULE BACTERIA OF THE LEGUMINOSAE

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In Bergey's Manual of Determinative Bacteriology, second edition, 1925, the root-nodule bacteria of the Leguminosae are classified in the genus *Rhizobium*, and two species *Rh. leguminosarum* and *Rh. radicicola* are recognized. Differentiation between the two species is established upon the basis of morphology, with particular reference to flagellation, cultural characters, and habitat of the organisms. Only incomplete descriptions of the organisms are given, which fact renders an accurate classification and identification difficult. With the exception of the habitat, no characteristic is described in sufficient detail to render identification possible, and only a very limited number of host plants are mentioned under each bacterial species.

In the seventh edition of Lehmann and Neumann's Bakteriologische Diagnostik, 1927, the nodule bacteria of the various leguminous plants are classified as one species, *Bacterium radicicola* Beijerinck. The possibility of recognizing several subspecies or varieties is mentioned.

At present both American and foreign investigators use several different names for the same organism. Scientific workers have not agreed, either as to the number of species which should be recognized, or as to the terminology which should be applied to them. To avoid confusion many have abandoned the use of scientific terminology and have substituted such terms as "root-nodule bacteria of legumes" or, when a more specific designation was desired, such terms as "alfalfa-nodule bacteria," "soybean-nodule bacteria" or "alfalfa bacteria," "soybean bacteria." At

best this can be regarded only as a temporary expedient and not as a solution of the question.

Certain other investigators have used the scientific terminology, e.g., *Rhizobium leguminosarum*, *Rh. radicicola*, *Bacillus radicicola*, or *Pseudomonus radicicola* without specifying the plant species from which the organism was secured. The inclusion of organisms differing as widely in their morphology and physiology as the root nodule organisms from alfalfa and bean in a single species *Rhizobium leguminosarum*, as is done by Bergey, leads to confusion. This is particularly true in such reports on the physiology of these microörganisms as that of Werkman, 1927.

The evidence in favor of the use of *Rhizobium* Frank as a generic term for this group seems quite conclusive to the writers. Since Buchanan (1926) has recently given an excellent review of the literature dealing with the synonymy of this group and has presented the facts regarding the use of this term, it is unnecessary to cover this material again, *Rhizobium* was included in the list of names recommended by the Committee of the Society of American Bacteriologists on Characterization and Classification for adoption by the Society; Bergey has adopted the term in his Manual of Determinative Bacteriology; and it has been commonly used by many scientists.

The genus *Rhizobium* was placed in the Family *Nitrobacteriaceae* and the Tribe *Azotobactereae* by the Committee of the Society of American Bacteriologists on Characterization and Classification of Bacteria, and by Bergey in his Manual of Determinative Bacteriology. Löhnis and Hansen, 1921, and many others have criticized this classification of the root nodule bacteria, and have insisted that the organisms are closely related to the colon-aerogenes and the colon-typhoid groups. In the opinion of the authors the view of Löhnis is the more nearly correct one. Certainly the root nodule organisms of the Leguminosae are very similar to certain of the plant pathogens in their morphology, physiology and cultural characteristics: e.g., *Phytomonas tumefaciens*, of the family *Bacteriaceae* and the tribe *Erwineae*. Also, the genus *Rhizobium* has very little in common with the autotropic bacteria of the family *Nitrobacteriaceae*.

The writers suggest that it would be advisable to transfer the genus *Rhizobium* from the *Nitrobacteriaceae* to the *Bacteriaceae* and place it in a new tribe standing close to the *Erwineae* and the *Bactereae*. *Hyphoidees* was suggested by Vuillemin, in 1905, since it drew attention to the "false hyphae" or "infection threads" formed in the nodule. Dangeard, 1926, used the term in his classification as a tribal designation. *Hyphoideae* would thus appear to be the proper designation for the tribe containing the genus *Rhizobium* and it is considered advisable to place this tribe in the family *Bacteriaceae*.

The early students in this field were for the most part of the opinion that all the organisms producing nodules on the roots of leguminous plants should be considered as a single species, although many investigators recognized that certain important differences were apparent among the representatives from the different plant species.

Frank, in 1879, noted certain differences between *Lupinus* and *Lathyrus* as to the type of nodule produced and the organisms contained in the nodule. He believed them all to belong to a single species and applied the name, *Schinzia leguminosarum*. Prior to this, 1877, Frank had stated that the fungus of the root nodules was *Schinzia cellulicola*.

Schroeter, in 1886, was the first to make a division of these organisms into two species. He transferred them from the genus *Schinzia* into a new genus *Phytomyxa*, and recognized two species: *Phytomyxa leguminosarum* (Frank) Schroeter and *Phytomyxa lupini* Schroeter. He said that the first species forms nodules on most leguminous plants, *e.g.*, *Trifolium repens*, *Lotus corniculatus*, *Orobus vernus*, etc.; while the second forms nodules on *Lupinus luteus* and *L. angustifolius*.

Beijerinck, in 1890, reported that the differences between the organisms of the root nodules were greater than he had earlier supposed, and that *Bacillus Ornithopi*, from *Serradella*, should be recognized as distinctly different from *Bacillus Fabae*, from *Vicia faba*. This is apparently his first use of the species designation *Bacillus Fabae*. In earlier papers and also in the same article he referred to this organism as *Bacillus radicicola* var. *Fabae*.

In 1888, he had recognized that not all the organisms are identical, and had listed seven varieties of *Bacillus radicicola*, namely varieties *Fabae*, *Vicia hirsutae*, *Trifoliorum*, *Pisi*, *Lathyri*, *Lupini*, and *Cytisi*, as well as mentioning *Phaseolus* and *Robinia*.

Schneider in 1892, described and named five species, with two varieties of one: *Rhizobium mutabile*, *Rh. curvum*, *Rh. Frankii*, varieties *majus* and *minus*, *Rh. dubium*, and *Rh. nodosum*.

Gonnermann, in 1894, secured cultures of several organisms from nodules of leguminous plants which were classified into two species with several varieties of each. Discarding all the earlier nomenclature, he named his organisms *Bacillus tuberigenus* and *Micrococcus tuberigenus*.

Kirchner, in 1895, studied the organism from soybean nodules and concluded that this organism is distinct from the others. The name *Rhizobacterium japonicum* was applied to it.

Many other cases might be cited in which the early scientific workers recognized that not all the nodule organisms of leguminous plants should be classed as belonging to a single species.

At the present time most workers agree that at least two species should be recognized, and a considerable number believe that sufficient differences exist to make necessary the creation of several species. As examples of the second group, recent papers by Dangeard, 1926, and Müller and Stapp, 1925, may be mentioned. Müller and Stapp, on the basis of the morphological, cultural, and physiological characters of the organisms, have separated eleven subgroups of the nodule bacteria of the Leguminosae. No scientific names have been used, nor species designations made. The authors hold, however, that it is possible definitely to identify each subgroup, and suggest that they should be classified on this basis. Dangeard, basing his work largely on careful studies of the nodules, decided that at least 10 species should be recognized. These are given names as follows: *Rhizobium Trifolii*, *Rh. polymorphum*, *Rh. Fabae*, *Rh. Meliloti*, *Rh. Loti*, *Rh. simplex*, *Rh. torulosum*, *Rh. Phaseoli*, *Rh. minimum*, *Rh. Sojae*.

Various workers have formed their classifications on the basis of either one, or a combination of more than one of the following

characters: (*a*) morphology; either on culture media or in the nodule; (*b*) cultural characters; type of colony and growth upon laboratory media; (*c*) physiology; as exhibited in the phenomena of plant inoculation, in the laboratory upon culture media, and in serological reactions. These characters are generally recognized at present as legitimate bases for species-differentiation.

Robson (1928), in his book entitled "The Species Problem" has brought out some worth-while suggestions regarding speciation. He considers that species in bacteria are probably of the same order as in higher animals. But they may well be less permanent from a human point of view and that with the admission of criteria other than morphological, pathogenicity becomes a more valid ground for specific differentiation.

Perhaps the most nearly constant of the physiological properties of the root-nodule bacteria is their ability to cause the formation of nodules upon certain species of the Leguminosae and not upon others. Certainly, from the practical standpoint, this is one of the most important characters of the organism. For more than twenty-five years, a utilitarian classification into "cross-inoculation" groups has been in use by students in this field. In the opinion of the writers, this characteristic is the most logical and valuable one on which to base any differentiation of the genus *Rhizobium* into species. The character which is now universally used to separate the members of this genus from other bacteria is the ability to form nodules on the roots of leguminous plants. If a further classification is to be made of the members of this genus, should not this character be used as a species identification? Detailed studies have shown, also, that the members of each "cross-inoculation" group differ from those of each of the others, in some or all of the following points: morphology, cultural characters, physiological properties, and serological reactions.

It may well be questioned whether the differences which are recognized are of a magnitude to justify specific or only varietal distinctions. To date no one in the field of bacteriology has been able to define clearly the characters upon which specific separa-

tions are to be based. As in all other fields of natural science, the judgment of the student, based upon, and in conformity with, similar cases, must be the deciding factor. In general, bacteriologists have agreed that a clear and definite difference in some essential character is a just and reasonable basis for species-separation. It is considered that the differences exhibited between the "cross-inoculation groups" are of that magnitude. Also further confusion must arise if the "cross-inoculation group" is to be recognized as a variety instead of a species, for it is clearly recognized that the members of the different "cross-inoculation groups" are further differentiated into subgroups based on cultural, physiological, and serological characters.

In view, therefore, of the fact that the root-nodule organisms naturally fall into several groups, each of which presents definite and constant characters differentiating it from all other organisms, and because of the confusion arising from the present system of classification, it is proposed that several separate species be established.

In the present proposal species-identification is based upon the morphology, cultural characters, physiological properties, and serological reactions of the bacteria. Many detailed reports have been published covering these characteristics, and only a few of the more outstanding points will be mentioned at this time. For more complete descriptions reference is made to the papers of Burrill and Hansen, 1917, Löhnis and Hansen, 1921, Wright, 1925, Stevens, 1925, Müller and Stapp, 1925, and Baldwin and Fred, 1927. Many others might be mentioned. In each case in which sufficient study has been made to render it possible, a species is established for each "cross-inoculation group." In deciding upon the nomenclature appropriate for these species, the authors have been guided by the rules of the International Code of Botanical Nomenclature.

1. *Rhizobium leguminosarum* Frank. The organism causing the formation of nodules upon the roots of *Lathyrus*, *Pisum*, *Vicia* and *Lens*.

a. Growth on mannitol agar is rapid with tendency to spread. Streak is raised, glistening, semi-translucent, and white. Con-

sistency is slimy and occasionally viscous. Considerable gum is formed.

b. Fermentation of carbohydrates. Slight acid-production from glucose, galactose, mannose, lactose and maltose.

c. Morphology. Peritrichous flagellation. Bacteroids from nodules are commonly irregular, with many X and Y shaped forms. Vacuolated forms predominate.

The species designation "*leguminosarum*" was proposed by Frank in 1879, when he erroneously placed the nodule forming organisms in the genus *Schinzia*. The greater portion of his work, as judged by his figures, was done with *Lathyrus* and *Orobus*, and *Orobus* is now placed in the genus *Lathyrus*. Frank recognized only the one species in the genus *Rhizobium*. If new species are to be established, the old species designation *leguminosarum* must be retained, and it would seem appropriate to apply it to the species including the organism causing the root nodules on *Lathyrus*.

2. *Rhizobium trifolii* Dangeard. The organisms causing the formation of nodules upon the roots of *Trifolium* sp.

a. Growth on mannitol agar is rapid. The colonies are white, becoming turbid as they grow older. Often the cultures become so mucilaginous that long threads may be drawn when the growth is touched with a needle. Streak cultures at first show a transparent growth along the line of inoculation. Later this growth becomes mucilaginous and flows down the inclined surface of the agar accumulating as a slimy mass at the bottom. Produces large amounts of gum.

b. Fermentation of carbohydrates. Slight acid—production from glucose, galactose, mannose, lactose and maltose. Usually slightly greater than with *Rh. leguminosarum*.

c. Morphology. Peritrichous flagellation. Bacteroids from nodules are pear-shaped, swollen, and vacuolated. Rarely X and Y shaped forms.

Beijerinck, in 1888, proposed *trifoliorum* as a varietal name for the organism isolated from *Trifolium*. Dangeard, in 1926, used the species term *trifolii*.

3. *Rhizobium phaseoli* Dangeard. Causes the formation of nodules on *Phaseolus vulgaris*, *Ph. angustifolia*, and *Ph. multiflorus*.

a. Growth on mannitol agar is rapid with tendency to spread. Streak is raised, glistening, semi-translucent, white. Consistency slimy and occasionally sticky but not so marked as in *Rh. trifolii*.

b. Fermentation of carbohydrates. Very slight acid-fermentation of glucose, galactose, mannose, sucrose and lactose.

c. Morphology. Peritrichous flagellation. Bacteroids from nodules are usually rods, with few branched forms. They are usually smaller than in *Rh. leguminosarum* and *Rh. trifolii*, and often vacuolated.

Beijerinck, in 1888, was able to distinguish the organisms of Phaseolus nodules from others and applied the term *Bacillus radicicola*, *phaseolus*-type. He did not, however, give it a definite varietal name. Schneider, in 1892, was the next to propose a specific designation for this organism. He proposed the term *Rh. Frankii* var. *majus* for the organisms symbiotic with *Phaseolus vulgaris*. *Rh. Frankii* var. *minus* was proposed for those symbiotic with *Pisum sativum*. Schneider's terminology is invalid from two standpoints. He failed to utilize any of the earlier specific and varietal names. His characterizations of the organisms are so meagre and conflicting that it is impossible to know definitely the organisms with which he worked. Dangeard, in 1926, used the term *Rh. phaseoli*.

4. *Rh. meliloti* Dangeard. The organism causing the formation of root nodules upon *Melilotus*, *Medicago*, and *Trigonella*.

a. Growth on mannitol agar is fairly rapid but not as fast as that of *Rh. leguminosarum*, *Rh. trifolii*, and *Rh. phaseoli*. Growth is moderate to abundant. The streak is raised, glistening, opaque, and pearly white. Consistency is buttery with considerable gum but usually not viscous.

b. Fermentation of carbohydrates. Strong acid-production from glucose, galactose, mannose and sucrose.

c. Morphology. Peritrichous flagellation. Bacteroids from nodules include both club-shaped and branched forms.

Dangeard first proposed this name in 1926, as applied to the organism symbiotic with members of the alfalfa-sweet clover

cross-inoculation group. Schneider in 1892 proposed the name *Rh. mutabile* for the organisms symbiotic with *Trifolium pratense*, *T. repens*, *Melilotus alba*, and *Lathyrus odoratus*. Schneider's terminology is invalid because of his failure to observe the rules of priority, and because of meagre and conflicting descriptions, rendering identification uncertain.

5. *Rh. japonicum* (Kirchner) comb. nov. The organism causing the formation of root nodules on *Soja max*.

a. Growth on mannitol agar is slow and scant under ordinary conditions. Streak is slightly raised, glistening, opaque, and white. Consistency is buttery with little gum formation. Pentose sugars give better growth than the hexoses.

b. Fermentation of carbohydrates. Little if any acid-formation. After prolonged incubation acid from xylose and arabinose.

c. Morphology. Monotrichous flagellation. Bacteroids of nodules are long, slender rods with only occasional branched and swollen forms.

This specific designation for the organism of the soybean root nodules was first proposed by Kirchner in 1895.

The five species described above comprise the list which is proposed at the present time. Specific designations for the organisms from the nodules of other leguminous plants are not proposed now, because of insufficient study and lack of definite information. As the study of these organisms continues, it will undoubtedly be desirable to increase the list of species.

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